The Journal of CLINICAL ENDOCRINOLOGY AND METABOLISM

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THE ACCUMULATION OF RADIOACTIVE IODINE BY HUMAN FETAL THYROIDS*

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THE increasing availability of radioactive iodine for treatment of thyrotoxicosis emphasizes a need for establishing limits of safety, especially in regard to the therapy of thyrotoxicosis in pregnant women. Chapman (1) and his group studied the uptake of radioiodine (I^{131}) in human fetal thyroids following its administration to the mothers. From the radioactivity of the macerated fetal thryoids, they concluded that accumulation of radioiodine by the human fetal thyroid was minimal until after the fourth month.

Our thyroid clinic group has maintained a cautious attitude toward radioiodine therapy of pregnant patients. Consequently we have not used I^{131} to treat any patient with thyrotoxicosis during a recognized pregnancy. In order to obtain additional clinical information, we are attempting to determine the age at which the human fetal thyroid begins to accumulate I^{131}.

METHODS

Through cooperation of the departments of Obstetrics, Internal Medicine, and Radiation Research, patients requiring therapeutic abortion were

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given 500 microcuries of I^{131} approximately twenty-four hours before surgical intervention. Immediately after the operation the fetuses were photographed and the thyroid glands removed in a block of tissue including the trachea. The radioactivity of the thyroid area was measured in a special counter (Texas Tank). Then the tissue was fixed for histologic study. Routine hematoxylin-eosin sections and radioautograph slides were prepared from each specimen. Maternal thyroidal uptake of the radioiodine was determined in most instances at twenty-four hours. The age of the fetuses was judged in two ways. First, by measuring their crown-rump length on the photographs and then referring to tables and nomographs of size-age relationships (2, 3). Second, by the history, in which the age was computed as the time since the mother's last normal menstrual period minus two weeks—on the assumption that conception was about fourteen day after that date.

RESULTS

Fetuses from 9 pregnancies, ranging in duration from 6½ to 15 weeks, were studied by this technique. There was demonstrable radioactivity in the thyroids of all but 3 (Table 1). Stable iodine had previously been given to 2 of these 3 mothers, presumably accounting for the failure of I^{131} accumulation. The third fetus was the youngest, 6½ weeks of age, and the thyroid was still primitive (Fig. 2, Fetus IV).

In addition to these euthyroid patients, 1 thyrotoxic patient who con-

TABLE 1

Fetus No.	Length (mm.) C-R	Estimated age (wks.)		Radioiodine uptake by thyroid		
		by length	by history	of mother* %	of fetus (Texas Tank)	Autograph
V	115	15	17	—	0.40	Pos.
I	113	15	14	30	0.10	Pos.
II	85	13	11	26	0.15	Pos.
III	85	13	8	29	0.00	Pos.
VIII	80	12½	12	—	0.00†	Faint
IX	76	12	14	30	0.01	Pos.
VII	73	12	11	22	0.00	Pos.
VI	42, 44 twins	8	10	—	0.00†	Neg.
IV	28	6½	7	46	0.00	Neg.

* By external count.

† Administration of stable iodine prior to I^{131}.

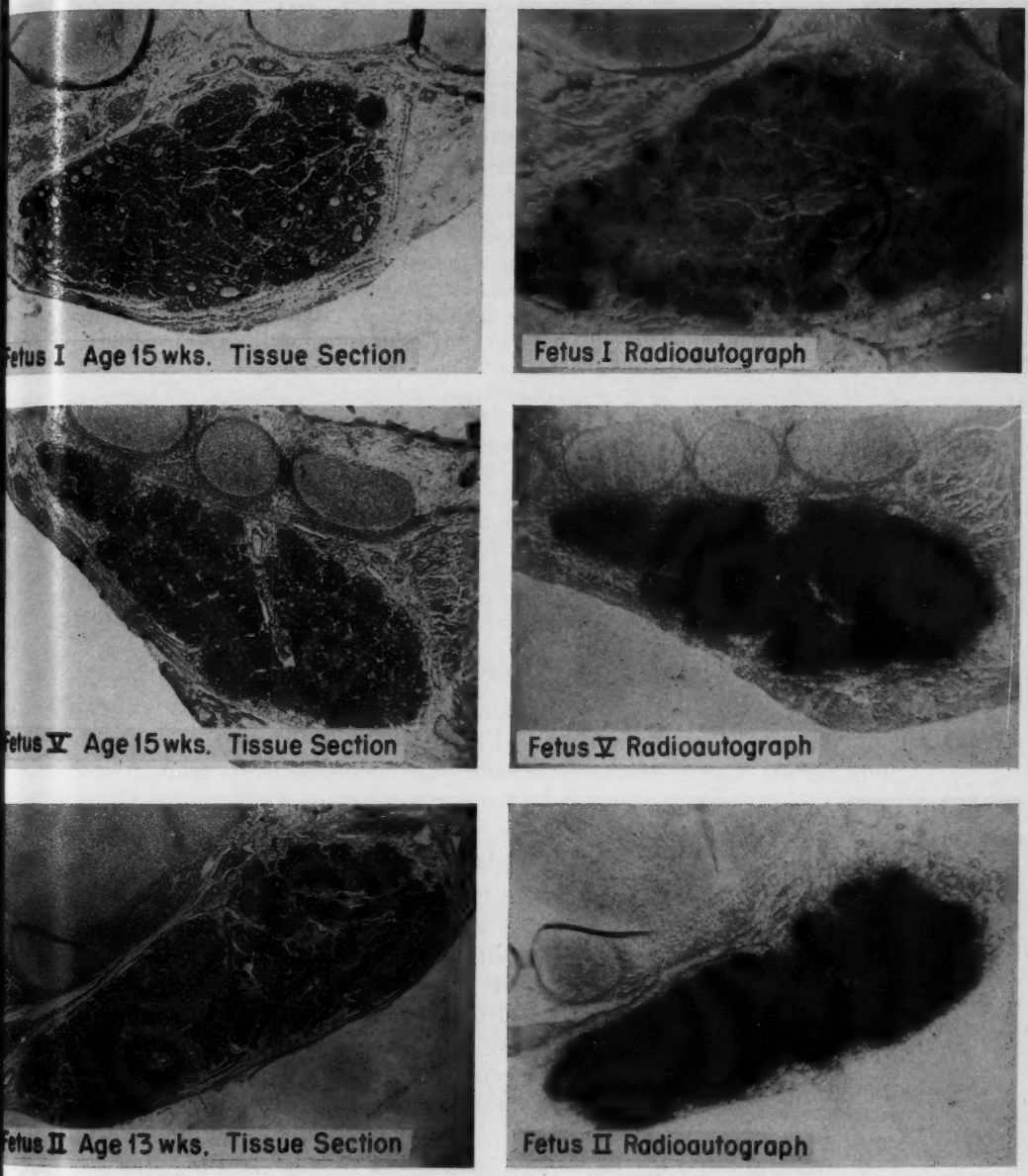
Fetus I Age 15 wks. Tissue Section
Fetus I Radioautograph
Fetus V Age 15 wks. Tissue Section
Fetus V Radioautograph
Fetus II Age 13 wks. Tissue Section
Fetus II Radioautograph

FIG. 1. Tissue sections (left) and radioautographs (right) of thyroid glands from Fetuses I, V and II (×90). Note abundant accumulation of radioactivity and beginning follicle formation at the periphery, in the thyroid of Fetus I.

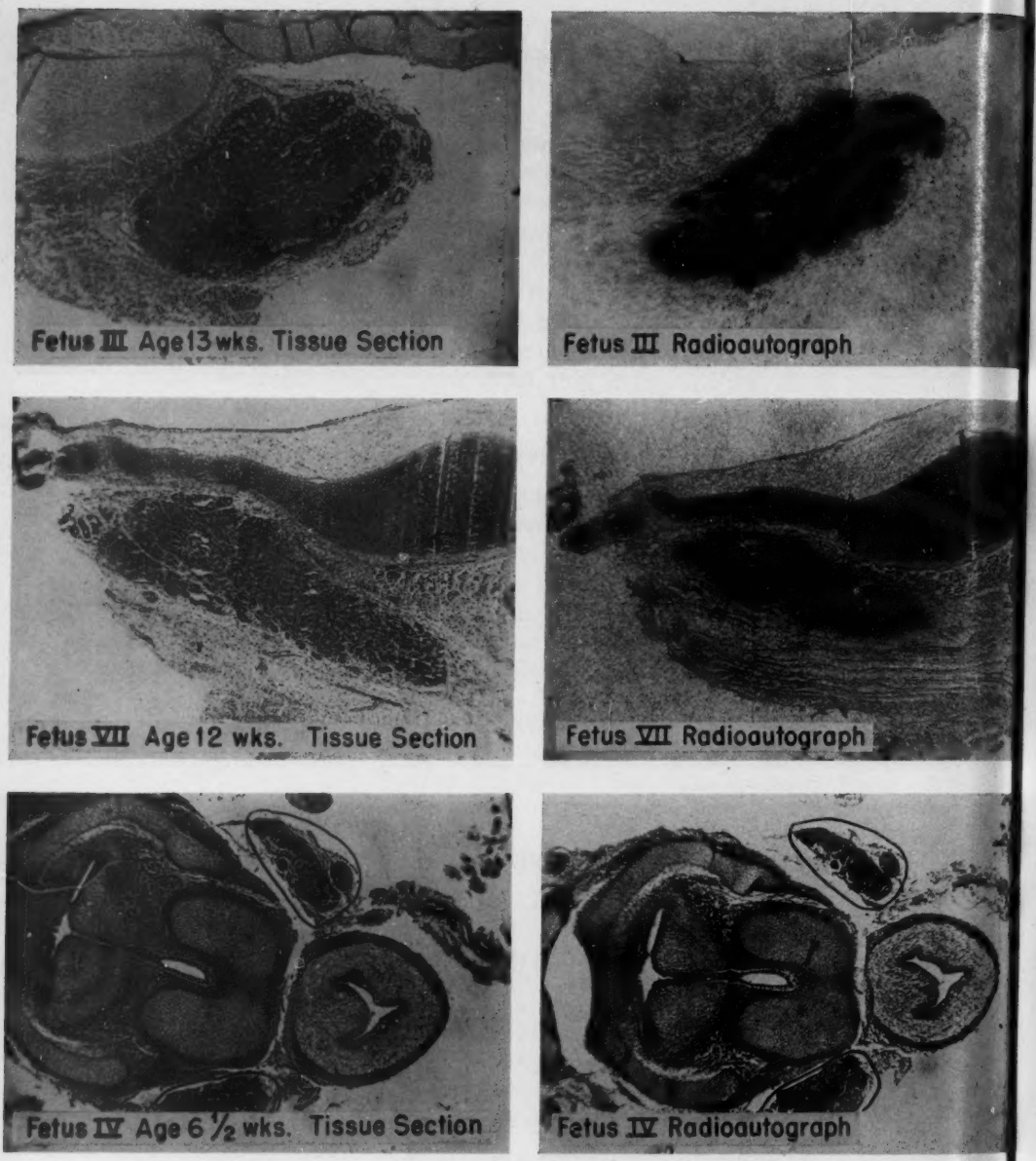
Fetus III Age 13 wks. Tissue Section
Fetus III Radioautograph
Fetus VII Age 12 wks. Tissue Section
Fetus VII Radioautograph
Fetus IV Age 6 ½ wks. Tissue Section
Fetus IV Radioautograph

FIG. 2. Tissue sections (left) and radioautographs (right) of thyroid glands from Fetuses III, VII and IV (×90). Note presence of radioactivity in the thyroids of Fetuses III and VII, but complete absence in the thyroid of Fetus IV. Fetus IV was so small that the esophagus and trachea are included in the fields; the thyroids are outlined in black.

cealed the fact that she was pregnant, was inadvertently treated with I^{131}. An outline of her history follows:

A.D., a 25-year-old housewife, para 4, gravida 6, was seen in the outclinic because of symptoms of nervousness, weight loss, tachycardia, and tremor for the preceding several months. Examination revealed marked hyperkinesis, a fine tremor of the hands, an overactive heart, and a diffuse goiter. Laboratory studies confirmed the diagnosis of thyrotoxicosis. Her record did not mention either amenorrhea or uterine enlargement, but subsequent events revealed that she must have been 4 weeks pregnant. She was given Tapazole in anticipation of thyroidectomy, and advised to return at regular intervals. She failed to take this medication, and did not return until four months later, at which time she manifested severe thyrotoxicosis. Her basal metabolic rate was +43 per cent, the serum protein-bound iodine concentration was 15.4 micrograms per 100 ml., and the radioiodine uptake of her thyroid gland was 83 per cent at twenty-four hours. She was treated with 6,100 microcuries of radioiodine, retaining 69 per cent at twenty-four hours. She was requested to return in three months for revaluation of her thyroid status. She was admitted to the Obstetrical Service at that time and a review of her history revealed she had been in the nineteenth week of gestation at the time of radioiodine therapy. The patient had become euthyroid. Three weeks later, labor was induced prematurely and the patient was delivered of a female infant weighing 2,125 grams. At 4 days of age the child had a serum protein-bound iodine level of 4.6 micrograms per 100 ml. A tracer dose of I^{131} was then administered and the child's thyroid took up 17 per cent at twenty-four hours. These are normal values for a newborn. At 1 year of age a pediatrician judged the child to be normal in height and weight and in good health. The concentration of serum protein-bound iodine was normal.

DISCUSSION

Our results indicate that the human fetal thyroid has the ability to accumulate demonstrable amounts of I^{131} by the twelfth week of gestation. The findings are in agreement with those of Chapman *et al.*, if crown-rump lengths are used for comparison rather than estimated fetal ages.

Since the thyroid glands were not dissected out cleanly for weighing, it was not possible to estimate the local density of irradiation in these fetal glands. It is a matter for conjecture whether this accumulation of iodine is enough to preclude I^{131} therapy of maternal thyrotoxicosis. Experimental studies in cattle (4) with a total gestation period of 285 days, have indicated that uptake of iodine occurs in the fetal thyroid as early as the fifty-third day of gestation. Late in bovine pregnancy, radioiodine was found to be six or seven times more concentrated in the fetal than in the maternal thyroid (5). However, these studies offer no information on whether radiation damage to the thyroids would have occurred had the animals been allowed to survive.

Chapman has reported 2 examples, and we have a third, of thyrotoxic mothers who were given therapeutic doses of radioiodine at a stage of pregnancy when the fetus presumably could accumulate iodine, and yet normal babies were born. Doubtless other pregnant women have been similarly treated since 1948. Two possible explanations are considered to account for

this lack of fetal thyroid damage. The first is that the quantity of I^{131} was insufficient to be harmful. In this series the uptake was demonstrable only by radioautograph. Furthermore, in thyrotoxicosis, the maternal thyroid has a much greater avidity for iodine, and hence might reduce the amount available to the fetus. In the present study, the maternal uptake was 69 per cent in the thyrotoxic patient as compared to 22 to 46 per cent (average 30 per cent) in the euthyroid women.

A second possibility is the fact that the concentration of circulating thyroxine is elevated in thyrotoxicosis and, according to Peterson (6), thyroxine can readily pass the placental barrier. Since thyroxine depresses the radioiodine uptake of the thyroid gland, the fetal uptake may be thus depressed.

Further studies are needed to determine the amount of radioiodine accumulated at any given age by the human fetus, and the sensitivity of the fetal thyroid to irradiation (7).

SUMMARY

The human fetal thyroid has the ability to accumulate demonstrable amounts of I^{131} by the twelfth week of gestation. It is a matter for conjecture whether this accumulation of iodine is enough to preclude I^{131} therapy of maternal thyrotoxicosis.

A case is reported of a thyrotoxic mother who received a therapeutic dose of I^{131} at a stage of pregnancy when the fetus presumably could accumulate iodine, and who was delivered of a normal baby near term. One year later the child was normal in height and weight, and was in good health.

Acknowledgments

The authors wish to express their gratitude to Drs. J. H. Randall and William C. Goddard for providing case material, to Dr. Francis Ritchey and Mr. Walter McGinn for assistance in preparing radioautographs, and to Drs. Emil Witschi and Jack Davies for determinations of fetal age.

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DYSGENESIS OF THE THYROID GLAND AS A CAUSE OF CRETINISM AND JUVENILE MYXEDEMA

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IN 1888 the Committee appointed by the Clinical Society of London (1) to study myxedema concluded that the disease described under the name of myxedema, as observed in adults, was practically the same disease as that named sporadic cretinism when affecting children. The Committee noted that although these conditions appeared to depend on, or to be associated with, destruction or loss of function of the thyroid gland, the ultimate cause of such destruction or loss was not evident. It would be equally true today to say that the ultimate cause of most cases of either adult or childhood myxedema or of sporadic cretinism, remains obscure. Obvious exceptions are: i) the hypothyroidism which follows accidental ingestion or therapeutic use of goitrogenic substances, including drugs; ii) endemic cretinism, which is usually goitrous and is due to lack of iodine; and iii) sporadic goitrous cretinism, which is usually familial and is probably due to an inborn error of metabolism, genetically transmitted (2, 3).

Means (4) urges that "distinction be made between juvenile myxedema, a disease acquired during childhood, and true cretinism, which is essentially a congenital abnormality." The better therapeutic prognosis in juvenile myxedema than in sporadic cretinism may in practice justify such a distinction. However, implicit therein are the opinions that juvenile myxedema and sporadic cretinism are separate conditions, probably with different etiologies, and that in juvenile myxedema a state of athyreosis develops in a previously normal child, whereas in cretinism athyreosis exists from the time of intra-uterine life or early infancy.

In the final analysis, the causes of sporadic cretinism and juvenile myxedema are unknown. The development of juvenile myxedema is usually ascribed to primary atrophy of the thyroid gland (the etiology of which is unknown), or very rarely to destruction of the gland, for example by thyroiditis or Hashimoto's struma. The etiology of sporadic cretinism is also obscure. It is held that it may be due to an embryonal lack of devel-

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opment of the thyroid gland or, more rarely, that it may result from destruction of the gland by thyroiditis during fetal life. Dorff's studies (5) on two pairs of twins, in which one twin in each pair was a sporadic cretin, favor the view that sporadic cretinism is due to a disturbance in the germ plasm of the anlage of the thyroid gland.

Encouraging experience with the use of radioactive iodine (I^{131}) in the investigation of nonendemic goitrous cretinism (3, 6) influenced us to apply similar techniques to the study of the problems of the etiology and interrelationship of so-called sporadic athyroidic cretinism and juvenile myxedema. The results of these studies in 4 children who presented the clinical features of hypothyroidism appear to us to be of sufficient interest and importance to warrant their publication. They support the opinion that hypothyroidism, at least in children, may on occasion be due to dysgenesis of the thyroid gland; that the same developmental defect may result in either juvenile myxedema or sporadic cretinism; and that the age at onset of the clinical features of thyroid insufficiency depends on the amount of thyroid tissue present in the fetus or child.

The techniques used in the I^{131} investigations have been described elsewhere (6, 7).

CASE REPORTS

Case 1

F.K. (Table 1), a boy aged 3½ months, was first admitted to the hospital on August 17, 1950. He was his mother's tenth child, the sixth by her second husband. The third child of the second family had been receiving treatment with desiccated thyroid when he died at the age of 6 months from meningitis. The patient was born spontaneously at full-term after a normal pregnancy, but was feeble and cyanosed. His birth weight was not known. He did not suck well and did not thrive. His bowels were constipated, and he was excessively lethargic.

At the time of admission his weight was 4.8 Kg. (normal, 5.3 Kg.) and his height 55 cm. (normal, 58.5 cm.). He had the typical appearance of a cretin. His face was pale and puffy, the skin coarse and dry, the hair dry, and the eyebrows deficient. Supraclavicular pads of fat were present. The hands were large and spade-like. The abdomen was protuberant, and there was an umbilical hernia. His cry was hoarse, and the tongue was large. No thyroid tissue was palpable in the neck.

The blood cholesterol level was 112 mg. per 100 ml. Radiographs of the wrists showed no centers of ossification.

Treatment with desiccated thyroid was started in a dosage of grain ¼, twice daily, and he was dismissed from the hospital on August 19, 1950. The dosage was gradually increased to grain 1, twice daily. His initial progress was satisfactory, but his mother proved unreliable and his supervision and treatment consequently became erratic. By September 14, 1950 he had lost his cretinous appearance. On November 30, 1950, a center of ossification was noted in the os magnum. At the age of 11 months he was sitting up well, and standing with support. At 16 months he was walking unaided. During his second year he had whooping cough, and in his third year measles and mumps. He was seen at the end of 1953 after an interval of six months, when he once again presented the

TABLE 1. CLINICAL AND LABORATORY DATA

	Case 1 F.K.	Case 2 M.S.	Case 3 S.M.J.	Case 4 G.A.
Sex	male	female	female	female
Age at onset of hypothyroidism	under 3.5 mo.	under 1 yr.	about 3 yrs.	under 13 yrs.
Age when studied with with I^{131}	3 yrs., 8 mo.	4 yrs., 3 mo.	7 yrs., 6 mo.	13 yrs.
Bone age at time of I^{131} studies	6 yrs. (after 3 yrs. of treatment)	under 1 yr.	3 yrs.	13 yrs.
Cholesterol of plasma (mg. per 100 ml.)	125	—	183	593
PBI^{127} of plasma (μg. per 100 ml.)	—	1.8	—	—
BMR (% of standard)	—	—	—	−32
Thyroid gland	not felt	not felt	not felt	not felt; thyroglossal cyst removed 9 mo. before
Response to desicc. thyroid orally	+	+*	+	+

* See footnote 1 (to case history of M.S.) in the text.

characteristic appearance of a cretin. It was obvious that treatment had lapsed. He was readmitted to the hospital on December 17, 1953. His weight was 11.8 Kg. (normal, 15.0 Kg.) and his height 82 cm. (normal 93 cm.). The cholesterol level was 125 mg. per 100 ml. Radiographs of the wrists showed advanced ossification equivalent to that of an average 6-year-old child.

During thyroid therapy, grains 2 daily, he once more lost his cretinous appearance. He became physically more active but remained mentally deficient.

Radioactive-iodine tests (Table 2). On January 19, 1954, he was given by mouth 35 μc. of carrier-free I^{131}. The accumulation of I^{131} in the neck was negligible, amounting to 3 per cent of the dose at twenty-four hours. Scanning of the neck by means of a lead-shielded Geiger-Müller counter (G.E.C., G.M. 4) showed no increased accumulation anywhere in the neck (Fig. 1). Estimation of the excretion of I^{131} in the urine was not practicable. Forty-eight hours after the dose of I^{131}, the total plasma radioactivity was 0.18 per cent of the dose per liter of plasma. The protein-bound fraction was nil.

Case 2

M.S. (Table 1), a girl aged 4 years and 3 months, was admitted to the hospital on

TABLE 2. RESULTS OF RADIOACTIVE-IODINE (I^{131}) STUDIES

	Case 1 F.K.	Case 2 M.S.	Case S.M.J.	Case 4 G.A.
Gland uptake of I^{131} at 24 hours:				
Per cent of dose	3	14	30	7
Localization				
Normal	none	none	none	none
Ectopic	none	sublingual	sublingual	sublingual
Urinary Excretion of I^{131} (% of dose):				
0–6 hrs.	—	—	20.0	23.3
6–24 hrs.	—	—	22.5	12.2
0–24 hrs.	—	52.8	42.5	35.5
24–48 hrs.	—	18.3	6.4	20.8
Blood I^{131} values at 48 hours (% dose/L. plasma):				
Total plasma I^{131}	0.18	0.89	0.84	0.89
Protein-bound I^{131}	Nil	0.28	0.36	0.12
Butanol-extractable I^{131}	—	0.06	0.28	0.10

April 30, 1954. One of twins, she had been born spontaneously after a normal pregnancy. She had an older sister and a younger brother, both of whom were well. Her twin, though smaller than the average for his years (93 cm. *vs.* the normal 100.5 cm.), underweight (13.6 Kg. *vs.* the normal 16.5 Kg.), and mentally retarded, presented none of the facial characteristics of a cretin; his ossification was within the normal limits for his age, and I^{131} studies showed good I^{131} concentration in a normally placed thyroid gland. Both parents were healthy. The mother had noticed enlargement of her own thyroid gland, diagnosed nontoxic, in May 1953.

The patient's birth weight was 4½ pounds (about 2 Kg.). Her development had always been unduly slow, even in relation to her twin brother. She cut her first tooth at 8 months, walked at 2 years, and talked at 3 years. Her bowels had always been constipated and she was unduly lethargic. She had had an umbilical hernia repaired.

At the time of her admission to the hospital the child weighed 12.3 Kg. (normal, 16.1 Kg.), and her height was 79 cm. (normal, 98.6 cm.). Her features were cretinous. She was fat, apathetic and somnolent. Her face was pale, her skin dry, and her hair sparse. She had a lumbar lordosis and a protuberant abdomen with the scar of a repaired umbilical hernia. The tongue was large. No thyroid tissue was felt in the neck. The plasma protein-bound iodine (PBI^{127}) level was 1.8 μg. per 100 ml. Radiographs of the wrist showed no centers of ossification. The electrocardiograph showed no definite deviation from normal: T was inverted in lead III.

Treatment with desiccated thyroid was started on May 20, 1954, and she became brighter and more active.[1]

[1] After this paper was submitted for publication it became obvious that this patient (M.S.) responded incompletely to desiccated thyroid. Although she became brighter and more active, she did not lose her myxedematous appearance and her ossification remained unaffected. She has since shown a completely satisfactory response to triiodothyronine.

Radioactive-iodine tests (Table 2). On May 10, 1954, she was given by mouth 40 μc. of carrier-free I^{131}. At twenty-four hours the accumulation of I^{131} in the neck was 14 per cent of the dose. Scanning of the neck showed that there was no thyroid tissue in the normal site, and that the only thyroid tissue present was in the region of the base of the tongue, just above the hyoid bone (Fig. 1). The urinary I^{131} excretion values were 52.8 per cent of the dose for the period 0–24 hours; and 18.3 per cent for the period 24–48 hours. The total plasma radioactivity forty-eight hours after the dose of I^{131} was 0.89 per cent of the dose per liter of plasma. The protein-bound iodine fraction was 0.28

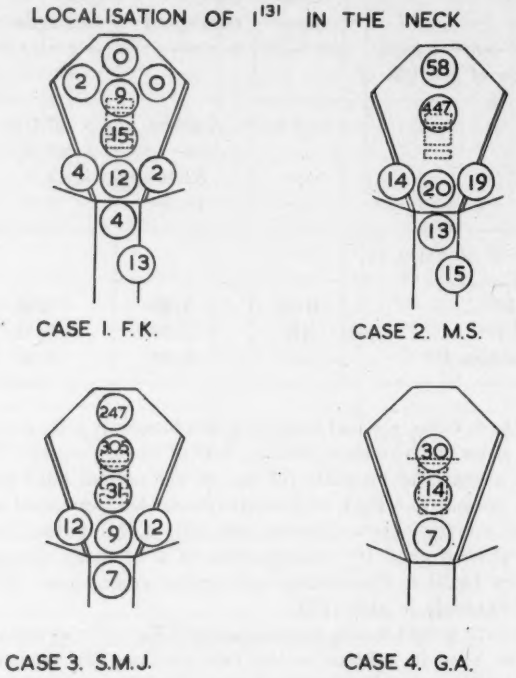

FIGURE 1

per cent of the dose per liter, and the butanol-extractable fraction was 0.06 per cent.

Details of Cases 3 and 4 have already been published (7).

Case 3

S.M.J. (Table 1), a girl aged 7½ years, was referred for medical opinion on November 30, 1953, because of failure to grow, especially since the age of 3 years. Her birth weight was 7 pounds, 14 ounces (about 3.6 Kg.). She walked and spoke single words at 15 months. Her bowels had always been constipated. She had done well at school.

Her weight was 17.6 Kg. (normal, 23.2 Kg.) and her height 104 cm. (normal, 119.2 cm.). Her skin was rather dry and she had an umbilical hernia. The body proportions were somewhat infantile, but the other features of myxedema were lacking. No thyroid tissue was felt in the neck.

The blood cholesterol level was 183 mg. per 100 ml. The electrocardiograph showed low voltage standard leads. Radiographs of the wrists showed the ossification of a child of 3 years.

The clinical response to desiccated thyroid, grains 2 daily, was excellent.

Radioactive-iodine tests (Table 2). On December 15, 1953, she was given by mouth 40 μc. of carrier-free I^{131}. At twenty-four hours the accumulation of I^{131} in the neck was 30 per cent of the dose. Scanning of the neck confirmed the absence of thyroid tissue in the normal site, and revealed the presence of a sublingual rest just above the hyoid bone (Fig. 1). In the periods 0–6 hours, 6–24 hours, and 24–48 hours, 20.0 per cent, 22.5 per cent and 6.4 per cent of the dose of I^{131}, respectively, were excreted in the urine. At forty-eight hours after the dose of I^{131} the total plasma radioactivity, protein-bound iodine fraction, and butanol-extractable I^{131} were 0.84 per cent, 0.36 per cent, and 0.28 per cent of the dose per liter of plasma respectively.

Case 4

G.A. (Table 1), a girl aged 13 years, was seen on October 15, 1954. Her birth weight was 10 pounds (4.5 Kg.). Her early progress was uneventful. At the age of 11 years a midline swelling was observed under the chin. It was removed in January 1953, under the mistaken impression that it was a thyroglossal cyst, but the histologic structure of the specimen was that of active thyroid tissue. After the operation it was noted that her speech became thick and slow, and her hair started to fall out. Mental activities and physical movements became slower, though her performance at school remained average.

Her weight was 30.6 Kg. (normal 41.4 Kg.) and her height was 127 cm. (normal 149.2 cm.). She had the coarse features, large tongue, dry scanty hair, and coarse cold skin of myxedema. No thyroid tissue was felt in the neck: there was a healed scar at the level of the hyoid bone.

The plasma cholesterol level was 593 mg. per 100 ml. The electrocardiograph showed low voltage in the standard leads with inverted T_3. Radiographs of the wrists showed no abnormality. The basal metabolic rate was −32 per cent.

During treatment with desiccated thyroid, grain 1 daily, the clinical improvement was excellent.

Radioactive-iodine tests (Table 2). On October 26, 1953, she was given by mouth 72 μc. of carrier-free I^{131}. At twenty-four hours the accumulation of I^{131} in the neck was negligible, namely 7 per cent of the dose. Scanning of the neck confirmed the absence of thyroid tissue in the normal site: scanty thyroid tissue was present in the area of the operation scar (Fig. 1). In the periods 0–6 hours, 6–24 hours, and 24–48 hours, 23.3 per cent, 12.2 per cent and 20.8 per cent of the dose of I^{131}, respectively, were excreted in the urine. At forty-eight hours after the dose of I^{131} the total plasma radioactivity, protein-bound iodine fraction, and butanol-extractable I^{131} were 0.89 per cent, 0.12 per cent, and 0.10 per cent of the dose per liter respectively.

DISCUSSION

Study of the neck accumulation of I^{131} in our 4 patients indicated that none had thyroid tissue in the normal site. F.K., who was clinically a sporadic athyroidic cretin, had no evidence of increased accumulation

anywhere in the neck. This negative finding may well indicate a complete absence of functioning thyroid tissue due to an agenesis of the thyroid gland. It is, however, impossible to exclude the transitory existence in utero of a small, albeit inadequate, focus of functioning tissue, which had undergone exhaustion atrophy or had atrophied after birth as the result of thyroid medication which had been given irregularly over a period of approximately three years. It is unlikely that thyroid treatment was the cause of the failure of extant functioning tissue to take up I^{131}, since an interval of at least two months had elapsed after the thyroid was stopped before the I^{131} studies were carried out. M.S., also, was classifiable on clinical and radiologic grounds as a sporadic cretin, though the onset of the clinical features had perhaps been more insidious than in F.K., and had aroused parental concern less early. S.M.J. was a hypothyroid dwarf, without the gross manifestations of myxedema. In G.A., who was almost certainly already hypothyroid before operation, the clinical features of myxedema developed when virtually all her thyroid tissue was removed by operation under the erroneous belief that a sublingual swelling was a thyroglossal cyst. M.S. and S.M.J. had foci of marked I^{131} accumulation in the neck above the hyoid bone, indicative of functioning thyroid tissue in the sublingual region. This tissue was undetectable clinically. The absence of any similar local accumulation at this site in 6 adult patients with myxedema, or in others who were euthyroid or hyperthyroid, precludes the possibility that the site of origin of the radioactivity might be the sublingual salivary gland. Furthermore, it has been our experience that when there is no increased accumulation of I^{131} in the neck, as in F.K., the 48-hour plasma protein-bound I^{131} (PBI^{131}) concentration is nil. The appreciable amounts of PBI^{131} in M.S. and S.M.J. are indirect evidence in support of thyroid tissue, not salivary gland, as the site of origin of the radioactivity. G.A. showed slight I^{131} accumulation in the area of the operation scar above the hyoid bone, and there was an appreciable concentration of PBI^{131} at forty-eight hours. These results indicate that she had scanty residual thyroid tissue.

Silverman and Wilkins (8) recently investigated the ability of hypothyroid children to concentrate I^{131} in the neck. In the patients with severe hypothyroidism dating from birth there was no uptake of I^{131}, whereas in patients in whom the onset of symptoms had been more insidious or had occurred at a later age there was a definite but low uptake. No attempt, however, was made to localize the site of I^{131} accumulation. The results of the 24-hour I^{131} uptake test in our patients F.K., M.S. and S.M.J. make an interesting comparison. In F.K., in whom the diagnosis of hypothyroidism was obvious shortly after birth, the 24-hour uptake was negligible; in M.S., in whom the evidence of hypothyroidism became manifest in the

first year, it was 14 per cent; and in S.M.J., in whom the hypothyroidism developed insidiously at about the age of 3 years, the 24-hour uptake was 30 per cent. G.A. is not suitable for comparison; in her, the development of evidence of hypothyroidism was more insidious than in the other patients, but virtually all her thyroid tissue had been removed before the I^{131} studies were made.

In retrospect, it is impossible to know if F.K. had had at one time scanty thyroid tissue which had eventually undergone exhaustion atrophy in a manner similar to that in which the frustrated hyperplastic gland of the iodine-deprived endemic goitrous person may eventually atrophy (9). Certainly when he was investigated at the age of 3 years there was no evidence of any functioning tissue. M.S. still had functioning thyroid tissue sublingually at the age of 4 years, but the appearance of the clinical features of cretinism in infancy indicates that this tissue had been inadequate to meet the increased metabolic demands incident to independent life (10). It would have been instructive to know if either of these patients, but particularly F.K., had had evidence of thyroid insufficiency, such as retarded bone development, at birth (5). The deficiency of tissue in S.M.J. and G.A. was less severe. Indeed, it may be assumed that the production of thyroid hormone had, for a time, been adequate for their needs. Ultimately, however, the body's demands outgrew the productive capacity of the available thyroid tissue, and signs of hypothyroidism appeared. Even when the ectopic thyroid tissue in G.A. enlarged enough to produce a visible swelling (probably the result of excessive stimulation with thyrotropin secreted in response to a state of hypothyroidism) it failed to compensate for the fundamental lack of tissue, and to supply enough thyroid hormone. A similar explanation probably accounts for the thyroid insufficiency which is likewise common in patients with lingual goiters (11–13), and which gives rise during periods of special stress to most of the simple hypertrophies of lingual thyroid tissue, especially in patients who have no demonstrable thyroid tissue in the neck. In these patients the ectopic thyroid tissue has been recognized only when it has hypertrophied sufficiently to produce local symptoms and signs. In a few instances its nature has been confirmed preoperatively by the uptake of I^{131}. In only 1 other instance are we aware of the detection by I^{131} of sublingual thyroid tissue when its presence was unsuspected clinically. This occurred in a 9-year-old boy with retarded physical and mental development, in whom there was also complete absence of functioning thyroid tissue in the normal thyroid region (14).

We agree with Dorff (5) that the time of appearance of the clinical features of thyroid insufficiency depends on the amount of functional thyroid tissue available to the fetus or child. It is our belief that such a

conception of a quantitative deficiency of thyroid tissue as a cause of hypothyroidism—in our experience due to maldevelopment of the thyroid gland, manifested by the presence of the sublingual accumulation of I^{131}—may apply not only in sporadic cretinism but also in juvenile myxedema. Dorff (5) expressed the opinion that there is "an independent, low grade fetal thyroid activity which is not directly influenced by the maternal thyroid hormone," and that "there is probably no transmission of thyroid hormone through the placenta from either the mother or the fetus." This is in direct contradiction to the view expressed in most text-books and first put forward by Kocher (15), namely, that the mother's thyroid gland can act vicariously for that of the fetus. However Dorff's views receive support from more recent observations. Chapman *et al.* (16) have shown increasing localization of radioactive iodine by the thyroid glands of human fetuses after the fourteenth week of pregnancy. Thyroxine has been demonstrated in the human embryo at the third month (17, 18). Smith (10) concluded that "the human organism seems able to provide its own thyroid hormone independently of the maternal supply for some time before birth." McClendon and McLennan (19) showed that the concentration of thyroid hormone iodine was less, often much less, in the umbilical than in the maternal circulation, which would suggest that thyroid hormone, if it does cross the placental barrier, does not do so unimpeded. It may reasonably be argued that the evidence we have cited supports Dorff's view of an independent fetal thyroid activity rather than excludes the transmission of thyroid hormone through the placenta from the mother to the fetus. We cannot but agree. We do suggest, however, that the presence in the fetus ultimately doomed to cretinism, of a scanty focus of functioning thyroid tissue, clinically undetectable (as in M.S.) but adequate for the needs of the fetus in utero, affords a likely explanation of the apparent normal appearance at birth but rapid development of cretinism after birth, when the metabolic needs increase. It is a more likely explanation than the unsubstantiated belief that the mother supplies the fetus with a store of thyroid hormone to tide it over its early postnatal period.

The presence of appreciable amounts of PBI^{131} in the plasma of hypothyroid patients warrants only brief comment here. This finding has been discussed in detail elsewhere (3, 7). It is our experience (unpublished) in adult myxedema that when neck scans reveal no I^{131} accumulation, the serum PBI^{131} at forty-eight hours is nil; and when there is definite I^{131} accumulation in the neck, there may be an appreciable amount of PBI^{131}. It is obvious that a similar state of affairs holds in children when one compares the absence of PBI^{131} at forty-eight hours in F.K. with the values in M.S., S.M.J. and G.A. (0.28, 0.36 and 0.12 per cent of the dose of I^{131} per liter of plasma respectively). The most attractive explanation of the

amounts of PBI^{131} found in the plasma of hypothyroid patients with scanty thyroid tissue is that put forward by Riggs (20), elaborated by Blom and Terpstra (21), and cited by Wayne (22). It envisages the very rapid production of inadequate amounts of hormone with an unusually large number of its molecules labelled with I^{131}, so that PBI^{127} values are low but PBI^{131} values are normal or increased. The PBI^{127} level was low (1.8 μg. per 100 ml.) in the only one of our patients (M.S.) in whom we were able to have it estimated, so that the hypothesis would fit the facts of the case.

CONCLUSIONS

The results of I^{131} studies in M.S., S.M.J. and G.A. suggest that the hypothyroidism, be it cretinism or juvenile myxedema, resulted from dysgenesis of the thyroid gland, the maldevelopment being characterized by failure to descend and impaired function. The analogy of the undescended thyroid with the undescended testicle, already made by Means (4), is apt.

The explanation, which might be suggested in F.K. and M.S., that the destruction of the thyroid gland by a thyroiditis during embryonic life was the cause of the hypothyroidism, is not very convincing and is untenable in M.S. It begs the question why, under the same environmental conditions, the thyroid gland of one twin was selectively destroyed by an infection presumably transmitted via the placenta, and that of the other twin showed no ill effects (5). It ignores the lack of evidence of any contemporary infection in the mother, and the absence of other residual stigmata in the child, and it fails to explain the ectopic site of the surviving thyroid tissue.

As a result of our experience we are of the opinion that at least some cases of sporadic cretinism and juvenile myxedema have a common origin in maldevelopment of the thyroid gland. The times of appearance of the clinical features of thyroid insufficiency depend on the amount of functional thyroid tissue available to the fetus or child.

The hypothesis that scanty thyroid tissue, which is adequate for normal development of the fetus in utero may prove inadequate when metabolic needs increase at birth, with the result that the child becomes hypothyroid in infancy, is an attractive alternative to the unconfirmed belief that the mother supplies the fetus with a store of thyroid hormone to tide it over its early postnatal period.

SUMMARY

1. The commonly accepted views on the etiology of myxedema and cretinism and on the interrelationships of juvenile myxedema and sporadic cretinism are presented.

2. The clinical data and the results of I^{131} studies in 4 hypothyroid children are recorded.

3. The I^{131} results suggest:

a. That hypothyroidism in children may, on occasion, be due to dysgenesis of the thyroid gland. In 3 of the series of 4 patients, the only thyroid tissue was sublingual; in 1 patient no thyroid tissue was detected.

b. That the same developmental defect may result in either cretinism or juvenile myxedema.

c. That the age at which the thyroid insufficiency manifests itself depends upon the amount of thyroid tissue present.

4. The hypothesis is advanced that the normal appearance of the cretin at birth may be due to the presence of a scanty, clinically undetectable, focus of functioning thyroid tissue, which is adequate for the metabolic needs in utero, but which proves unequal to the increased metabolic requirements of independent life.

5. Brief consideration is given to the explanation of the presence of appreciable amounts of protein-bound I^{131} in the plasma of hypothyroid patients with scanty thyroid tissue.

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MATERNAL HYPERPARATHYROIDISM AS A CAUSE OF NEONATAL TETANY:

CASE REPORT AND REVIEW OF 29 CASES OF PARATHYROID ADENOMA

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VERY few reports of hypocalcemic tetany in the newborn associated with parathyroid overactivity in the mother have appeared in the literature. The first case was that of Friderichsen in 1938, in which medical investigation of a young woman was prompted by the presence of hypocalcemia and tetany in her infant (1). It is interesting that in this instance tetany did not develop until the infant was 5 months old, at which time cow's milk was substituted for breast milk. Only 3 other cases have been recorded, the most recent being that of Walton, in which neonatal tetany occurred in 2 siblings (2, 3). Similar observations have been made after the administration of Parathormone to gravid rats (4).

This is a report of neonatal tetany in the third child of a 30-year-old woman whose hyperparathyroidism was unsuspected until almost four years later.

CASE HISTORIES

R.W., the mother, aged 34, was admitted to the Medical Service of Presbyterian Hospital in April 1953, with a five-year history of polyuria, dysuria, recurrent chills, and flank pains. In 1948, she had been admitted to the Urology Service with left sided renal colic, vomiting, and fever. At that time the findings on physical examination were not remarkable. Intravenous and retrograde pyelograms were normal, and no calculi were seen. Determinations of serum calcium and phosphorus concentration were not made. Following discharge, her symptoms continued intermittently, and in 1952, one year prior to the final admission, the first of four calculi had been passed, preceded by renal colic. One calculus, analyzed at another hospital, was said to contain calcium. Recurrent episodes of renal colic on the left and right sides, malaise, urinary frequency, and occasional low-grade fever continued until the patient revisited the Urology Clinic in March 1953, her first return to this medical center in four years. The serum calcium

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level was found to be elevated, and the patient was admitted to the Medical Service. Upon further questioning, the patient stated that her third child, born four years previously at this medical center (Sloane Hospital for Women), had had severe convulsions shortly after birth, but following treatment he had been well. His case record is reviewed subsequently. Two other children, born in 1943 and 1947, had no similar symptoms.

Physical examination: A healthy-appearing white female; blood pressure 150/90; although a careful examination was made of the neck, no abnormalities were found.

Laboratory examination: Urine—normal, except for rare hyaline casts. Blood—Hgb. 14.5 Gm. per 100 ml.; red cells 5,090,000 and white cells 7,950 per cu. mm.; erythrocyte sedimentation rate 14 mm. in one hour (Westergren); serum concentration of nonprotein nitrogen 27 mg., albumin 4.2 Gm., globulin 2.6 Gm., calcium 15 mg., phosphorus 2.4 mg. and alkaline phosphatase 8 Bodansky units per 100 ml. An intravenous pyelogram was normal, and roentgenograms of the skull, shoulder, spine, pelvis, hips, and long bones were normal.

Surgical exploration of the neck was performed on May 4, 1953 and a parathyroid adenoma was removed, which measured 4.5×1.6×0.8 cm. Mild tetanic symptoms occurred on the fourth postoperative day but subsided quickly when oral calcium and vitamin D were started. Recovery was uneventful, and therapy was soon stopped. Since then the patient has remained asymptomatic.

C.W., the infant son, was born May 10, 1949, weighed 5,095 Gm. at birth[1] and was apparently normal. He was started on an evaporated cow's milk formula and at the age of 6 days was noted to have persistent tachypnea; he was therefore transferred from the Sloane nursery to the Babies Hospital. The findings on physical examination were negative, the cerebrospinal fluid was normal, and the serum calcium level was 7.7 mg. per 100 ml.

At 9 days of age, the child was discharged; no specific therapy was given. Within twenty-four hours progressively severe convulsions and muscle spasms occurred, so the infant was re-admitted. At that time, the spinal fluid was again normal, the serum calcium level was 7.5 mg., phosphorus 10.6 mg. and alkaline phosphatase 14.2 Bodansky units per 100 ml.

Convulsions were terminated promptly following intravenous injection of calcium gluconate. With oral calcium chloride and vitamin D therapy, the child became asymptomatic, the serum calcium level rose to 11.0 mg. per 100 ml., and one month later treatment was discontinued. Since then he has remained asymptomatic and has developed normally, except for poor dentition.

DISCUSSION

Although the presence of a parathyroid adenoma in this woman was not suspected for almost four years following the appearance of hypocalcemia in her third infant, the onset prior to this pregnancy of persistent symptoms which did not disappear until after removal of a parathyroid adenoma make it almost certain that hyperparathyroidism had been present at the time of the pregnancy.

In the light of the work of Gardner and others (5), the fact that the in-

[1] In spite of this heavy birth weight and a family history of diabetes mellitus, the mother's glucose tolerance has remained normal.

fant had a high phosphate intake in the form of cow's milk undoubtedly aggravated and hastened the onset of tetany. This case can be compared to that of Friderichsen (1), in which breast feeding prevented the appearance of symptoms until the fifth month of life. On the other hand hypocalcemic tetany has been reported in newborn infants who were breast-fed and whose mothers had normal serum levels of calcium and phosphorus (6). Great variations in individual responsiveness must, therefore, occur. However, there has been no documented case report of an asymptomatic infant being born of a mother with primary hyperparathyroidism.

As was adequately discussed in an earlier report (3), there is little definitive evidence concerning the causative agent in neonatal hypocalcemia, since excessive levels of calcium and of parathyroid hormone are each capable of directly inhibiting the activity of the parathyroid gland. The induction of hypocalcemic tetany by an inorganic phosphate load does not involve parathyroid inhibition; in fact, administration of phosphate is believed to result in parathyroid hypertrophy (5). Evidence that parathyroid hormone cannot cross the placental membrane (7) awaits confirmation before this mechanism can be disregarded. However, since calcium is known to have an inhibitory effect (8), and since it crosses the placental barrier easily (9), there is little reason in the present case to incriminate the high maternal level of parathyroid hormone. The agent responsible for the hypocalcemic neonatal tetany which occasionally occurs in the absence of primary hyperparathyroidism in the mother is more difficult to determine. In pregnancy the serum calcium concentration is normal or low and, although the level of parathyroid hormone has been shown to be elevated by the thirty-fourth week of pregnancy ("physiologic hyperparathyroidism"), it apparently drops to normal before term (10).

A review of the case records of the Presbyterian Hospital in New York and the King County Hospital in Seattle, revealed 28 additional cases of hyperparathyroidism in which a parathyroid adenoma was demonstrated at operation. The purpose of this survey was to attempt to find further instances of unrecognized neonatal tetany. However, it was immediately apparent that in no case except the one presented had pregnancy occurred within three years of the onset of symptoms (Table 1). The average age at onset of symptoms in the females of this group was 41 years—young enough so that more instances of pregnancy in hyperparathyroid women might normally be expected. It may be that the presence of hyperparathyroidism is not conducive to pregnancy. Indirect support for this possibility is found in the apparent high incidence of stillbirths and abortions noted by others (3), although not evident in our series.

It has been stated (11) that pregnancy contributes to the development

TABLE 1. PATIENTS WITH PROVEN PARATHYROID ADENOMA, PRESBYTERIAN HOSPITAL AND KING COUNTY HOSPITAL, 1930–1954

Age at onset of symptoms (yrs.)	*Minimum* number of years since last pregnancy	Number of cases
FEMALES		
36–49	Never pregnant	6
18	Adenoma removed before first preg.	1
29	Patient RW (this report)	1
25–29	three	2
30–34	four	2
35–39	three	3
40–49	eight	4
50–59	nineteen	5
MALES		5
		Total 29

of hyperparathyroidism, probably due to the parathyroid stimulation effected by relative hypocalcemia. The fact that one fourth of our hyperparathyroid female patients were never pregnant does not lend support to this hypothesis, though it in no way disproves it. The sex incidence in this series (24 females to 5 males) is similar to that observed in previous studies.

SUMMARY

A case of hyperparathyroidism due to a parathyroid adenoma is presented, in which diagnosis was not made until five years after the onset of symptoms, and four years following the birth of a child in whom neonatal tetany developed due to hypocalcemia.

In a review of 28 other cases with parathyroid adenomata no similar instances were found, possibly because in no other case had pregnancy occurred less than three years before the onset of symptoms.

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THE HISTOCHEMISTRY OF THE HUMAN PITUITARY BODY. I. PRELIMINARY REPORT, INCLUDING A DIFFERENTIATION BETWEEN BASOPHILS OF THE ANTERIOR AND POSTERIOR LOBES*

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THIS report presents preliminary results obtained by comparing consecutive sections of human pituitary glands stained with a variety of histologic and histochemical stains. This investigation has a twofold objective: 1) to determine the staining properties of the histologically defined groups of cells, and 2) to determine the homogeneity or lack of homogeneity of these cell groups, on the basis of cell-by-cell comparison of consecutive sections stained by different means.

Demonstration of specific cellular sites of formation of the pituitary hormones has not kept pace with the number of hormones isolated from this gland. This discrepancy may be considered as casting doubt on either the distinctness of the hormones isolated or the functional unity of the cell types as defined by staining properties. Doubt appears to be more justified in the latter instance, but the acidophils, basophils and chromophobes of the pituitary body have, in the main, resisted further breakdown.

That there are differences within at least one of the three classically defined groups of cells comprising the human anterior pituitary was first suggested by Romeis (1). He described two types of basophils noted after kresazan staining. More recently Pearse (2) used the periodic acid-Schiff reagent to demonstrate pituitary glycoprotein (3); he observed a higher proportion of basophils with this method as compared with conventional Mallory-trichrome preparations of similar sections.

Careful work on pituitaries of normal, gonadectomized and thyroidectomized rats by Halmi (4, 5) and Griesbach and Purves (6, 7) has resulted in the differential staining of two types of basophils in that species: thyro-

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tropes and gonadotropes, believed to be the sites of origin of the thyrotropic and gonadotropic hormones, respectively. These staining differences were achieved with the aldehyde fuchsin-trichrome method (8) in the normal rat and with both the periodic acid-Schiff reagent and the aldehyde fuchsin-trichrome method in the surgically treated animals.

Observations on the variation in staining properties of the cells of the human pituitary gland are presented in this preliminary report. Correlations between the specific microscopic findings and the clinical status of the donor will be presented in a subsequent paper.

MATERIALS AND METHODS

Both fetal and adult human hypophyses were studied. The fetal glands were obtained at routine postmortem examination of fetal and neonatal cadavers referred from this area to Dr. Edith Potter, Chicago Lying-In Hospital, over a one-year period beginning August 1, 1953. Over a corresponding period, adult glands were obtained in autopsy cases from the University of Chicago Clinics in which examination of the head was performed.[1] The maximum time between death and autopsy was eighteen hours for the adults; since the fetal group included stillbirths and abortions, such limits could not be established.

The pituitary glands were fixed in cold (4° C.) neutral formalin, kept at pH 6.4–6.6 by a phosphate buffer. After twenty-four hours the gland was trimmed and weighed. Adult glands were divided into five sagittal sections or, rarely, into four horizontal sections, washed for from six to twenty-four hours and embedded in paraffin (melting point: 56° C.) after acetone and alcohol dehydration. The fetal glands were embedded either intact, or as two horizontal sections. Microscopic sections were cut at 5 micra and consecutive sections mounted on serially numbered slides. Thus, on each slide, adult glands presented five sagittal sections or four horizontal sections; and fetal glands, one or two horizontal sections.

At least six staining methods were used for each gland studied. Direct comparison between cells was possible for adjacent slides because there were always consecutive sections. The three histologic stains employed were hematoxylin and eosin (H & E), periodic acid-Schiff (PAS), and aldehyde fuchsin-trichrome (AF). Esterases were demonstrated by the use of both naphthol AS acetate (AS) and alpha-naphthol acetate (α) as substrates (9); and alkaline phosphatase (alk. ϕ) by the alpha-naphthyl phosphate method (9).

The choice of these methods to demonstrate pituitary cells was based on one or more of three considerations: 1) their common use in studies reported in the literature, 2) the knowledge, or partial knowledge, of the underlying chemical reaction, 3) the different interpretations of the results. Thus the H & E and the PAS allow for comparison with the results of other workers; the naphtholic acetates, the naphthyl phosphate and the PAS methods demonstrate intracellular aliesterases, alkaline phosphatase and potential aldehyde substances (presumably glycoproteins), respectively. The AF method, for which the histochemical basis is not known (10), was used because of the separation that it affords between two types of basophils in the rat, and because of the vivid demonstration of the major cell types obtained when it is combined with Gomori's trichrome stain.

[1] The author acknowledges with thanks the kindness of the Pathology Department, whose members obtained these specimens.

A seventh stain, Klüver-Barrera Luxol Fast Blue (11), was used on a number of glands. The original basis for its trial was its unique phthalocyanin structure and its affinity for phospholipid-containing structures. Early trials demonstrated its specificity for acidophils, a not unexpected result when one considers the recent finding by Rennels (12) of similar localization of phospholipids in rat pituitaries stained by Baker's acid hematin technique (13).

In every case, sections were deparaffinized with xylol, then taken through alcohol and water. The naphtholic substrate mixtures for the AS and α techniques were prepared as described by Gomori (9), using Garnet Red GBC and Red RC as the diazonium salts; the substrate for the alkaline phosphatase method was that described by Gomori (9), and Red TR was the coloring agent used. All sections were then stained according to previously described methods; the sole alteration was the use of Wermel's modification (14) of Schiff's reagent for the PAS. The Klüver-Barrera stain was applied precisely as described for paraffin tissue in the authors' original report (11), except for Nuclear Fast Red rather than cresyl violet as counterstain. The counterstain in each of the first six methods was hematoxylin, with the addition of quinoline yellow in some PAS slides.

RESULTS AND DISCUSSION

Of the seven stains described, two (PAS, AF) have been reported to be specific for pituitary basophils (4–7); two others (AS and α-esterases) have manifested similar specificity in preliminary trials on human hypophyses (15). Abolins (16) has reported on the alkaline phosphatase reaction for demonstration of acidophils in the guinea pig. Table 1 presents the color

TABLE 1. COLOR CHARACTERISTICS OF HUMAN ANTERIOR-LOBE CELLS

	Acidophils	*Basophils*	*Chromophobes*
H & E	Pink	Bluish	Colorless
PAS	Colorless	Pink-red	Colorless
AF-trichrome	Red	Purple-blue*	Colorless
AS-esterase	Colorless	Bright red	Colorless
α-Esterase	Colorless	Brown	Colorless
α-Naphthyl PO_4	Colorless†	Colorless	Colorless
Luxol Blue	Bright blue	Colorless	Colorless

* In the rat, Halmi (4, 5) has shown that the basophilic cells which are stained by the light green component of the trichrome stain are delta cells, or gonadotropes. Such green cells are only rarely present in human glands; their occurrence will be discussed in a later report.

† In contrast to Abolins' (16) finding in the guinea-pig pituitary, none of the anterior-lobe cells of the human pituitary gland contains consistently demonstrable amounts of alkaline phosphatase. Additional studies by the present author on alcohol-fixed tissues and formalin-fixed frozen tissue, duplicating the conditions of Abolins' experiments, also failed to reveal significant amounts of alkaline phosphatase in anterior-lobe cells. However, we have repeatedly observed the almost exclusive localization of this enzyme to posterior-lobe blood vessel walls.

characteristics of the three major types of human anterior-lobe cells as revealed by these stains along with Luxol Blue and H & E.

Cell-by-cell comparison of many fields in more than 100 human pituitary glands demonstrates an almost perfect correspondence between the anterior-lobe cells labelled basophilic by each of the five methods just described. Plate I illustrates this finding, which was observed in virtually all postpubertal pituitary glands.

The most striking difference noted was in the staining of the cells invading the posterior lobe—the basophilic cells clustered in the posterior lobe in a large percentage of human adults. These cells behaved as basophils with H & E, PAS and AF staining. This was not the case when the AS- or α-esterase techniques were used. Only a small fraction of these cells demonstrated any AS- or α-esterase activity. This difference is presented in Plate II. It should be noted that whereas the basophils of the intermediate lobe approximate the invasive cells in their staining reactions, those of the anterior lobe do not.

Fetal and prepubertal pituitary glands were distinguished from those of adults in that the anterior-lobe cells of the former were not invariant with respect to stain. The AS- and α-esterase methods revealed only a fraction of those cells demonstrated to be basophils by the PAS and AF methods.

Results with the Klüver-Barrera stain demonstrated cells with a varying degree of conformity to that group revealed as acidophilic by any other stain. Although we have not observed staining of other cells than acidophils by Luxol Blue, the percentage of acidophils taking this stain varies widely from gland to gland—the correlation ranging from less than 10 per cent to almost 100 per cent. Superficially, this variation is analogous to that seen among red blood cells in the sections. However, the heterogeneity in the response of the acidophils differs from that of erythrocytes in two important respects: 1) the foregoing 100 per cent correlation is never observed in the case of erythrocytes, and 2) the staining of pituitary acidophils and the staining of erythrocytic elements in a given gland do not parallel each other.

The relation of these findings to functional differences among the cells of the anterior and intermediate lobes, in fetal and adult pituitary glands, is not within the scope of this preliminary report. The close histochemical relationship between the basophils of the intermediate lobe and the basophils invading the posterior lobe adds further support to the generally accepted intermediate-lobe origin of the latter.

It should be emphasized that MacCallum (17), in 1935, claimed that a modification of Cowdry's copper hematoxylin stain for mitochondria afforded a distinction between anterior-lobe and intermediate-lobe baso-

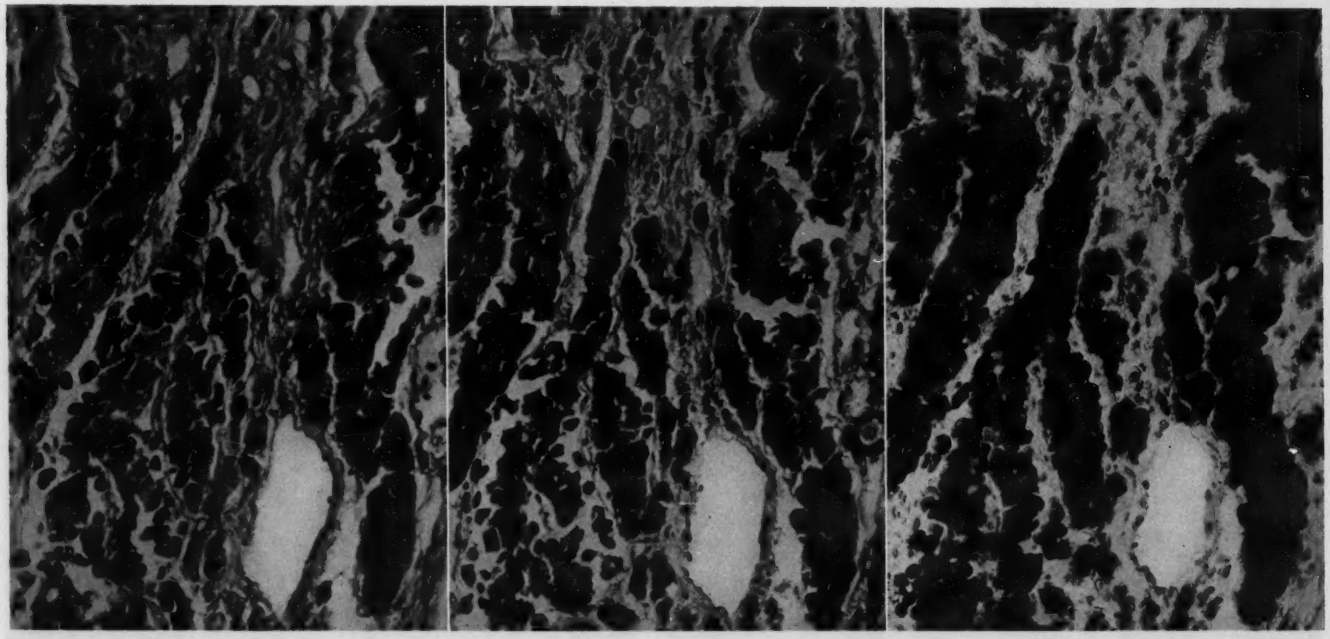

FIG. 1 FIG. 2 FIG. 3

PLATE I: Identical fields from consecutive sections of human anterior hypophysial lobe. Figures 1, 2 and 3 show staining with PAS, AF and AS methods, respectively. Note the exact correspondence among the basophils, which are black in these photographs. (×256)

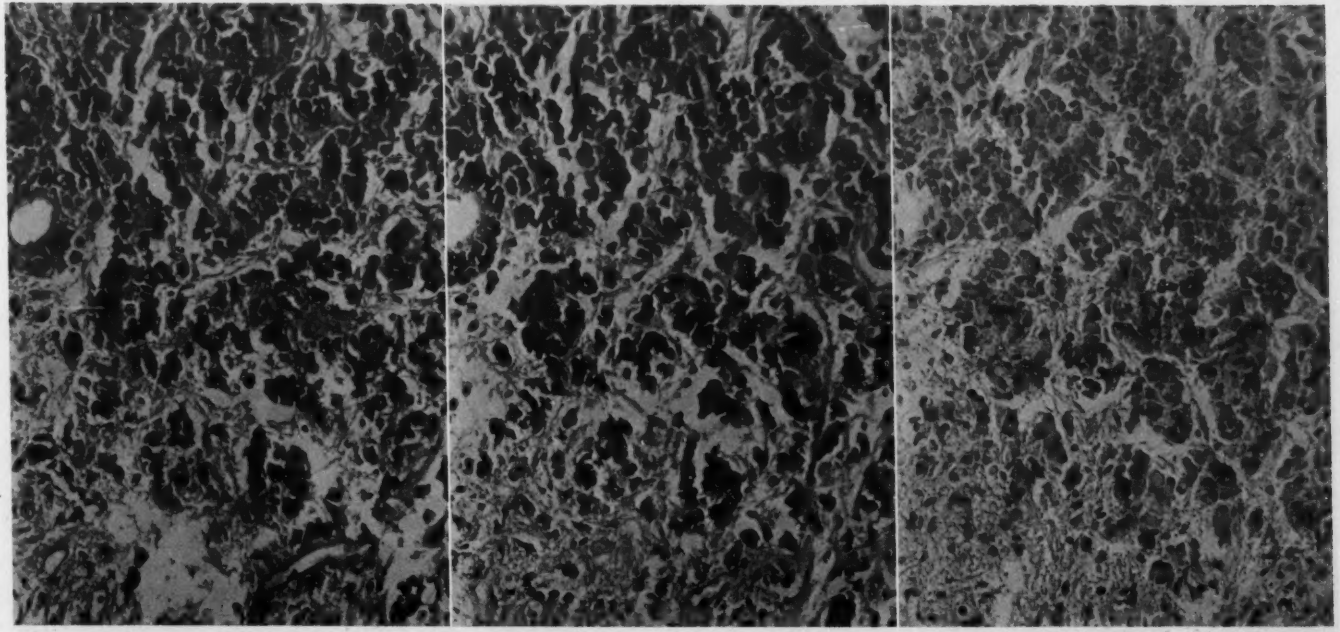

FIG. 4 FIG. 5 FIG. 6

PLATE II: Identical fields from consecutive sections of human posterior hypophysial lobe, at the site of the invading cells. Figures 4 (PAS) and 5(AF) duplicate each other (basophils black). Figure 6, stained by the AS method, demonstrates the negative reaction of these basophils for aliesterase, in contrast to the reaction of anterior-lobe basophils depicted in Figure 3. (×256)

phils. Although this method was properly reclassified as a positive stain for acidophils by Rasmussen in 1936 (18), it has continued to be used, erroneously, for distinguishing anterior-lobe and intermediate-lobe basophils (19). Even Mallory's excellent and much used text (20), republished six years after Rasmussen's refutation, lists the method described by MacCallum.

Copper hematoxylin has been observed by us to stain only pituitary acidophils. Because of common failure to obtain reproducible results in sections differentiated in borax-ferricyanide, it is not recommended even for acidophils. It may certainly not be used as a stain for the basophils of any lobe.

SUMMARY

Comparison of serial sections of human pituitary glands stained with hematoxylin and eosin, periodic-acid Schiff reagent, aldehyde fuchsin-trichrome and the aliesterase methods utilizing naphthol AS acetate and alpha-naphthol acetate as substrates, did not reveal any staining differences among the basophils of the anterior lobe of the adult.

Basophils which invade the posterior lobe may be differentiated from anterior-lobe basophils by means of the two aliesterase methods (AS- and α-esterase complements are either entirely absent or sharply reduced). The hypothesis that posterior-lobe invading cells have their origin in intermediate-lobe basophils is supported by these findings.

In the fetus, the histochemical complement of anterior-lobe basophils differs from that in the adult, when judged by the tinctorial response to aliesterase techniques. Some prepubertal glands exhibit such "fetal," esterase-poor cells.

The Klüver-Barrera copper porphyrin technique stains only a variable proportion of anterior-lobe acidophils. The reason for this is not clear, and artifacts due to fixation, dehydration and embedding have not been ruled out.

Further investigation of these findings is under way.

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PRELIMINARY INVESTIGATIONS INTO THE NATURE OF NEUTRAL 17-KETOSTEROIDS IN HUMAN PLASMA*

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INTRODUCTION

ALTHOUGH the quantitative and qualitative pattern of neutral 17-ketosteroids in the urine of normal individuals has been fairly well established, until recently, little has been known of these substances in the human blood. Gardner (1) has described a method for their determination in human plasma. He reported that adult males have levels of from 40 to 130 μg. per 100 ml. of plasma. Migeon and Plager (2), by means of paper chromatography, have isolated and identified dehydroepiandrosterone from human plasma. Another compound in smaller amounts was isolated, which was thought to be androsterone and/or etiocholanolone.

The study to be reported was undertaken in an attempt to define more clearly the pattern of neutral 17-ketosteroids in human plasma. A large volume of plasma was extracted so that there would be enough steroidal material for fractionation by means of column chromatography and identification by definitive means. This communication deals with a description and the results of such a study.

METHODS AND MATERIALS

A 950-ml. sample of plasma was obtained from 2,000 ml. of freshly drawn, heparinized whole blood. The blood was drawn from 4 healthy, adult males ranging in age from 23 to 36 years.

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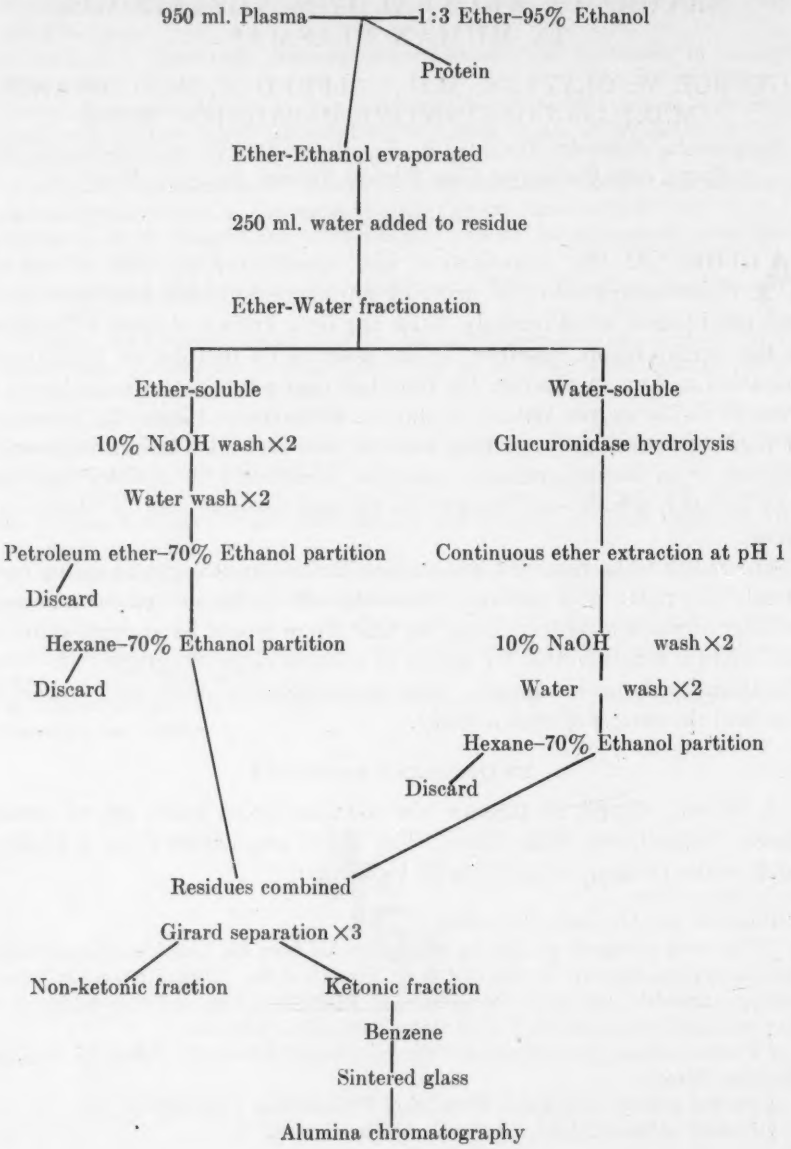
As outlined in the accompanying flow diagram, the plasma was immediately mixed with 7,000 ml. of 1:3 ether-95 per cent ethanol and this mixture was swirled for thirty minutes. The protein, which was precipitated by the ether-ethanol mixture, was then separated and washed with 7,000 ml. of the ether-ethanol mixture. The ether-ethanol extracts were combined and evaporated with an air blast and with gentle heat. The resultant heavy,



yellow, semi-liquid residue was taken up with 250 ml. of distilled water and 75 ml. of re-distilled ether. The mixture was gently shaken in a large separatory funnel and the ether drawn off. This procedure was repeated twice more. The ether extracts were combined and the aqueous fraction put aside.

1. *Ethereal fraction:* The volume of the ether extract was reduced to approximately 100 ml.; this was washed twice with 25 ml. of 10 per cent sodium hydroxide and twice with 25 ml. of distilled water. It was dried with sodium sulfate and evaporated to dryness with an air blast. The yellow, greasy residue was taken up in 70 ml. of absolute alcohol, warmed gently, and diluted with 30 ml. of distilled water. This mixture was shaken in a separatory funnel with 30 ml. of petroleum ether and the petroleum ether drawn off. The procedure was repeated twice. The petroleum-ether portions were then combined and extracted five times with 25-ml. portions of 70 per cent ethanol. All of the ethanolic fractions were combined and evaporated to dryness. The petroleum ether was discarded. The residue was again similarly partitioned between 70 per cent ethanol and hexane. The ethanolic fraction was evaporated and the residue put aside. The hexane was discarded.

2. *Aqueous fraction:* The pH of the aqueous fraction was adjusted to 4.5 with acetate buffer. After the addition of 50,000 units of β-glucuronidase, the mixture was incubated at 37° for forty-eight hours, at which time the pH was adjusted to 1.0 with 40 per cent sulfuric acid and extracted continuously with ether for forty-eight hours. The ether extract was reduced in volume and washed twice with 25 ml. of 10 per cent sodium hydroxide, then twice with 25 ml. of distilled water. The ether was evaporated to dryness and the yellow residue was partitioned between hexane and 70 per cent ethanol. The hexane was discarded and the residues of the original aqueous and ethereal fractions combined.

Girard separation: The Girard procedure was carried out on the crude neutral extract by a method similar to that described by Schneider (3). The residue was taken up in 80 per cent methanol and glacial acetic acid, followed by the addition of 100 mg. of Girard's reagent T. This mixture was warmed and put aside at room temperature for twelve hours. It was then chilled, diluted with ice water, and the nonketonic fraction removed by extracting rapidly with chloroform. The pH of the aqueous fraction, containing the ketosteroid hydrazones, was adjusted to 1.0 with 40 per cent sulfuric acid and the 17-ketosteroids were recovered by continuous ether extraction for forty-eight hours. The ether was washed with 10 per cent sodium hydroxide and distilled water, dried with sodium sulfate, and evaporated to dryness.

The chloroform, containing the nonketonic material, was evaporated to dryness and the Girard procedure was repeated twice more. The three ketonic fractions were then pooled and the Zimmermann test was performed on a 1/1,000 aliquot.

Chromatography: Chromatography was carried out on alumina which had been standardized according to the technique of Dingemanse (4). A straight glass tube, 7 mm. in diameter and 70 cm. in length, was employed. A tip was drawn out and plugged with glass wool. A 3-gram portion of alumina was carefully poured into the tube, which had been previously filled with redistilled benzene. The height of the alumina column was 12.5 cm. and the steroid : alumina ratio was calculated to be 1 : 1,840. The neutral ketonic fraction was taken up with benzene, filtered through sintered glass, and applied to the alumina column. The chromatogram was then developed by increasing concentrations of ethanol in benzene as 10-ml. eluents. Zimmermann reactions were carried out, in groups of five, on 1 ml. of each eluate. The polarity of the succeeding five eluents was determined by the amount of Zimmermann reacting material in each preceding five eluates. Table 1 shows the concentration of solvents and the order of 10-ml. eluents.

The Zimmermann reaction for the determination of 17-ketosteroids was carried out in a manner similar to that described by Gardner (1). Androsterone was used as a standard and the results recorded as micrograms of androsterone.

Determination of the "blue chromogens" was carried out on material from selected fractions, by the method of Allen (5), modified by using smaller quantities of reagents. Dehydroepiandrosterone was employed as a standard. The amount of "blue chromogen"

TABLE 1

Elution solvents (per eluate: 10 ml.)	Number of eluates
Benzene	1–4
Benzene+0.05 vol. per cent Ethanol	5–16
Benzene+0.1 vol. per cent Ethanol	17–36
Benzene+0.5 vol. per cent Ethanol	37–48
Benzene+1 vol. per cent Ethanol	49–53
Benzene+10 vol. per cent Ethanol	54 (20 ml.)

was estimated quantitatively by applying the equation for optical density derived by Allen (6):

$$\text{OD } 600 \text{ m}\mu - \frac{\text{OD } 560 + \text{OD } 640}{2}$$

Material from some of the fractions was dissolved in concentrated sulfuric acid and the absorption spectra determined. These data were compared to those of known steroids detailed by Bernstein (7).

Paper chromatography of certain of the residues was performed, using propylene glycol as the stationary phase and hexane as the mobile phase (8). Dehydroepiandrosterone was used as a standard.

The benzene used was thiophene-free and was redistilled over sodium before using. The ether was redistilled before use. The alumina was obtained from the Harshaw Chemical Company of Elyria, Ohio.

All determinations of pH were made on a Beckman pH meter. All absorption spectra were determined with a Beckman DU spectrophotometer.

RESULTS

Before chromatography, the Zimmermann test was performed on a 1/1000 aliquot of the total neutral ketonic fraction. The concentration of 17-ketosteroids was 171 μg. per 100 ml. of plasma extracted (total, 1.64 mg.). We have found that adult males have average levels of 187 μg. per 100 ml. of plasma (9). The sum of the values of the 54 fractions obtained by chromatography was 1.71 mg.

The results of the chromatographic analysis of the plasma extract is shown in Figure 1. It can be seen that three minor peaks and five major peaks are present. After chromatography the eluates which comprised these peaks were pooled into three minor fractions designated IA, IB and

IC, and five major fractions designated II, III, IV, V and VI. In addition, eluates 42 to 54 were pooled and designated as fraction VII.

Fraction I: None of the material in this fraction was identified. Fraction IA consisted of 48 μg. of Zimmermann-reacting material which had absorption maxima in sulfuric acid at 255–260 mμ [178][1] and 320 mμ [254]. Fractions IB and IC consisted of 55.9 and 52 μg. respectively. Both had absorption maxima at about 300 mμ [360] and minima at 250 mμ [307] in concentrated sulfuric acid.

Fraction II: This fraction was composed of four eluates having a sum

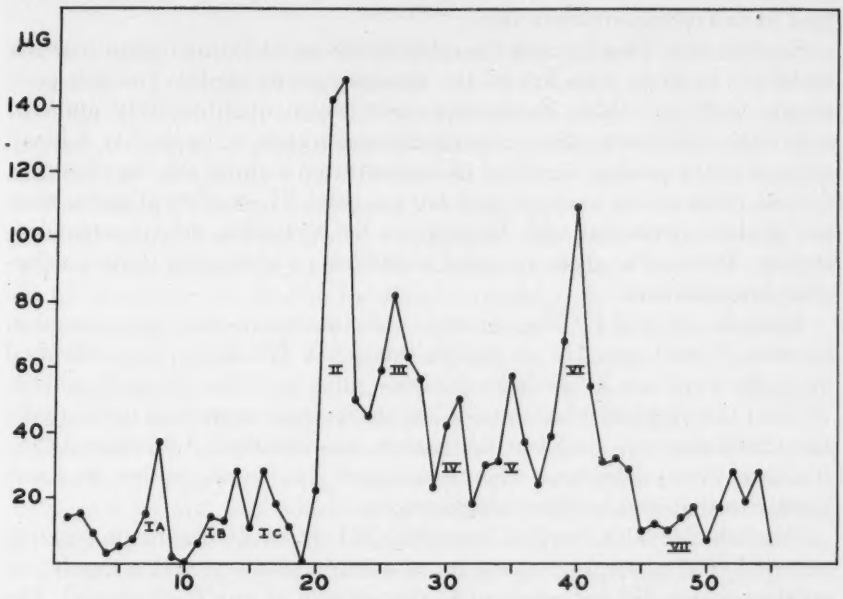


FIG. 1. Chromatographic analysis of plasma extract. (On the abscissa are shown the numbers of the eluents; on the ordinate, the amount of Zimmermann-reacting material in micrograms. See text for details.)

of 364 μg. of Zimmermann-reacting material. Material from each eluate gave a positive result with the "blue chromogen" test (absorption maximum at 600 mμ). Quantitatively the amount of "blue chromogen" material in each eluate approximated the amount of Zimmermann-reacting material. A small amount of material dissolved in concentrated sulfuric acid gave absorption maxima at 310 mμ [490] and 405 mμ [380]. These

[1] $\left(E\,\frac{1\%}{1\text{cm.}}\right)$

maxima are in agreement with those given by authentic crystalline dehydroepiandrosterone. Infrared analysis revealed that, although the absorption was strong, the spectrum was ill defined, due to contamination. The material was unidentifiable but there was a suggestion that part of it was acetylated. This acetylation may have occurred during the course of the Girard separation. The material was saponified with 5 per cent potassium hydroxide in ethanol and rechromatographed; however, the yield of Zimmermann-reacting material was too small for infrared analysis. When it was submitted to paper chromatography, a single band of Zimmermann-reacting material was found which had an R_T identical to that of dehydroepiandrosterone.

Fraction III: This fraction consisted of 358 μg. of Zimmermann-reacting material. Aliquots from five of the eluates making up this fraction gave results with the "blue chromogen" test which quantitatively appro. i-mated the results with the Zimmermann test in each of the eluents. A small amount of the residue dissolved in concentrated sulfuric acid had maxima of absorption at 308 mμ [460] and 410 mμ [400]. These absorption maxima are in close agreement with those given by crystalline dehydroepiandrosterone. Infrared analysis revealed a spectrum identical to that of dehydroepiandrosterone.

Fractions IV and V: The amount of Zimmermann-reacting material in fraction IV was only 115 μg. and in fraction V 173 μg. As the individual amounts were small, the material was submitted for infrared analysis without carrying out other tests. When the fractions were read individually the absorption was weak but the pattern was identical. Subsequently the fractions were pooled and, when reanalyzed, had an absorption spectrum similar to that of dehydroepiandrosterone.

Fraction VI: This fraction contained 301 μg. of Zimmermann-reacting material. The absorption spectrum in sulfuric acid revealed a number of maxima which did not conform to the pattern of any C-19 steroid. The infrared spectrum was suggestive of a mixture of dehydroepiandrosterone and androsterone in a ratio of 40:60. When the material was chromatographed on paper, two bands of Zimmermann-reacting material were found. The slower moving component had an R_T value identical to that of dehydroepiandrosterone and the faster moving band, the R_T of androsterone. Each component was determined quantitatively by eluting the steroids individually from the paper and carrying out the Zimmermann test on the eluates. The calculated amout of dehydroepiandrosterone was 104 μg. and that of androsterone, 197 μg.

Fraction VII: The remainder of the Zimmermann-reacting material (eluates 44 to 54) amounted to 215 μg. When paper chromatography was carried out, it was found in three bands. The fastest moving component

had an R_T identical to that of androsterone. The slower moving components had R_T values of 0.125 and 0.314 as compared to dehydroepiandrosterone. It was felt that these slow moving components probably represented 11-oxygenated 17-ketosteroids or 20-ketones.

A total of 1,114 μg. of ketonic material was identified or partially identified as dehydroepiandrosterone comprising 65 per cent of the total neutral ketonic fraction. The amount of androsterone was calculated to be 117 μg. or 11 per cent of the total neutral ketonic fraction; 24 per cent of the total was unidentified.

DISCUSSION

The process of purification and isolation of steroids from plasma presents a number of problems because the amount of steroid is small as compared with the other constituents. In our procedure the initial ether-ethanol extraction served to precipitate the plasma proteins, which make up a considerable part of the solid constituents, and probably to break steroid-protein bonds. The ether-water partition was performed primarily to divide the large amount of lipid which was present. The solvent partitions also reduced the amount of lipid and the Girard separation was of considerable aid in preparing the residue for chromatography. It is obvious that the chromatographic separation was not ideal, as manifested by the number of separate peaks made up of dehydroepiandrosterone and the failure to separate androsterone and dehydroepiandrosterone. However, it did serve to separate the major components into workable fractions.

The recovery of dehydroepiandrosterone as the predominant 17-ketosteroid in the plasma of adult males is in agreement with the observation of Migeon (2) and is of considerable interest. This compound is regarded by some (10) as a major intermediary metabolite of the adrenal cortex and hence would be expected to be found in sizable amounts in the peripheral blood. Studies have shown that dehydroepiandrosterone makes up only approximately 20 per cent of the total 17-ketosteroids in the urine of normal individuals (11), but both the studies of Migeon and those reported here reveal that dehydroepiandrosterone makes up more than 50 per cent of the total 17-ketosteroids in the plasma.

It has been found that if the 17-ketosteroids in plasma are fractionated into those which are hydrolyzed with glucuronidase and those which are hydrolyzed with acid, the 17-ketosteroid glucuronosides average 12 per cent of the total (9). The amount of unconjugated steroid in plasma is insignificant. It is well established that the urinary 17-ketosteroid glucuronosides make up approximately 50 per cent of the total and that the remainder can be hydrolyzed by acid (12). It is thought that the ketosteroid conjugates hydrolyzed by acid are mainly conjugated as sulfates.

The urine/plasma ratios of the various 17-ketosteroid conjugates are approximately as follows:

$$\frac{\text{urine glucuronosides}}{\text{plasma glucuronosides}} = \frac{50}{12} \qquad \frac{\text{urine sulfates}}{\text{plasma sulfates}} = \frac{50}{88}$$

From these ratios it appears that the excretion of 17-ketosteroid glucuronosides occurs with greater facility.

Eik Nes *et al.* (13) have found that the solubilities of steroids in certain protein solutions are greatest if the steroids are unconjugated. They have suggested that unconjugated steroids are firmly bound to protein and are poorly excreted, whereas conjugated steroids (more insoluble in protein solutions) are poorly bound to plasma protein and are rapidly excreted. Their studies did not include any differentiation between the solubilities of steroid sulfates and glucuronosides; however, from the differences in the rates of excretion which have been observed, it seems possible that the ketosteroid sulfate may be more firmly bound to protein and hence less easily excreted than the glucuronoside.

Munson *et al.* (14) have isolated dehydroepiandrosterone sulfate from the urine of normal individuals. Furthermore, it has been stated that dehydroepiandrosterone is almost entirely conjugated as a sulfate in human urine (15).

From the foregoing observations, one possibility may be suggested—that a greater proportion of dehydroepiandrosterone is found in the plasma because, as a 17-ketosteroid sulfate, it is more firmly bound to protein and less easily excreted. It cannot be denied that there are other possibilities which might explain the relative disproportion between the amount of dehydroepiandrosterone in plasma and the amount in urine. Other routes of excretion, such as the bile, may be of importance but these have not been thoroughly studied. The possible role of renal tissue in the conversion of dehydroepiandrosterone to other 17-ketosteroids should also be considered.

The inability to demonstrate material resembling etiocholanolone is puzzling, as it, along with androsterone, is present in equal quantities in the urine of normal adults. Etiocholanolone is known to be a metabolic product of testosterone as well as of a number of other adrenocortical steroids. In our experiments, perhaps it did occur in very small amounts which escaped detection.

The finding of small amounts of highly polar Zimmermann-reacting material, which probably represented 11-oxygenated 17-ketosteroids or 20-ketones, was not unexpected.

SUMMARY

A pool of 950 ml. of plasma from 4 healthy adult males has been examined for neutral 17-ketosteroid content. The procedure involved extraction of

the steroid with ether-ethanol, hydrolysis of the 17-ketosteroid conjugates, and purification by solvent partitions and Girard separation. The crude neutral fraction was chromatographed on alumina.

Dehydroepiandrosterone and androsterone were identified, the former constituting 65 per cent and the latter, 11 per cent of the total neutral ketonic fraction. Trace amounts of unidentified 17-ketosteroids were found. A possible explanation for these findings has been suggested.

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A METHOD FOR THE FRACTIONATION AND MEASUREMENT OF 17-KETOSTEROIDS IN HUMAN PLASMA*

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ANDROGENIC hormones are known to occur in human plasma. McCullagh and co-workers (1) found that ether extracts of 50 ml. of blood stimulated capon comb growth. Koch (2) confirmed these results. We recently reported the isolation of dehydroepiandrosterone (DHA) from normal human plasma (3). Romanoff *et al.* (4) reported the presence of both Δ^4-androstene-3,17-dione and 11β-hydroxy-Δ^4-androstene-3,17-dione in adrenal vein blood of subjects receiving ACTH. The second compound was more recently isolated by the same group of workers (5). Testosterone and Δ^4-androstene-3,17-dione were also isolated from spermatic vein blood of dogs by West *et al.* (6). It is reasonable, therefore, to attempt to measure 17-ketosteroids (17-KS) in human plasma.

Urinary 17-KS provide information concerning only the end-products of steroid metabolism. To study hormones produced by endocrine glands, one should examine the blood from the efferent vein of the considered glands, since later these hormones undergo various transformations throughout the organism. Therefore, the 17-ketosteroids of peripheral-vein plasma probably do not represent the true glandular products any more than do the 17-ketosteroids of urine. However, the assay of 17-KS may well be a valuable means of studying another phase of androgen metabolism.

We previously summarized and discussed (7) the methods described by Zimmermann (8), Dumazert and Valensi (9), West *et al.* (10) and Gardner (11). The Zimmermann reaction, the one most widely used for the determination of 17-KS, is not very specific. It is well known that many compounds of the fatty fraction of plasma extracts show this reaction. Such

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compounds are not 17-KS, as they have R_f values on our chromatograms higher than that of even the most simple 17-KS, androstane-17-one. Therefore, it is essential to deal with plasma extracts from which all fat has been eliminated.

It is the purpose of the present paper to present a method which is satisfactorily specific for 17-KS. It is through utilization of this method that we were able to isolate DHA from peripheral-vein plasma of human beings, thereby proving the specificity of the technique.

METHODS

A. *Reagents*

1. DuPont sulfuric acid, C.P.
2. Ether, U.S.P.
3. Sodium bicarbonate, 10 per cent solution.
4. Florisil (obtained from Floridin Company, Warren Pa.), washed with ethanol, dried and activated at 600° C. for four hours as described by Eik-Nes *et al.* (12).
5. Glass wool, washed with redistilled chloroform three times and dried at room temperature.
6. Chloroform, U.S.P., redistilled from K_2CO_3 immediately before use.
7. Methanol, U.S.P., twice redistilled.
8. Sodium hydroxide solution, 1 N.
9. Absolute ethanol redistilled from K_2CO_3.
10. Reagents for Zimmermann reaction: Absolute ethanol; 2.5 N KOH in absolute ethanol; 1 per cent twice recrystallized metadinitrobenzene (11) in absolute ethanol.

B. *Special equipment*

1. Continuous extraction apparatus with magnetic stirrers.
2. Constant-temperature room (35° C.) with tanks for paper chromatography.
3. Apparatus described by Bush (13) for elution of paper strips.
4. Beckman DU spectrophotometer with micro-attachment and microcuvettes.

C. *Hydrolysis*

Several methods were studied:

1. *No hydrolysis.* Some plasma was extracted directly without previous hydrolysis, in order to determine whether the 17-KS could be found in the free form.

2. *β-Glucuronidase hydrolysis.* The enzyme used in this work was a commercial beef-liver preparation stated to contain 5,000 units per ml. Our assay of this material by the method of Talalay *et al.* (14) showed that it contained 6,000 units per ml. To 100 ml. of plasma were added 62,500 units of β-glucuronidase and 50,000 units of penicillin. The pH was adjusted to 4.6 with acetate buffer and the mixture was incubated at 47° C. for fifteen hours. This procedure was an adaptation to plasma of the technique for urine reported by Glenn and Nelson (15).

3. *Hydrochloric-acid hydrolysis.* A 10-ml. portion of concentrated HCl was added to 100 ml. of plasma and the mixture was boiled on a hot plate for ten minutes.

4. *Protein precipitation with alcohol.* This is an adaptation of the method described by West *et al.* (10). To 100 ml. of plasma was added 500 ml. of 100 per cent ethanol; the proteins were removed and washed twice with 200 ml. of ethanol. The total ethanol extract was evaporated to near dryness. The residue was dissolved in 500 ml. of distilled water, and to this was added 50 ml. of concentrated HCl. The mixture was boiled on a hot plate for fifteen minutes and then rapidly cooled.

5. *Sulfuric-acid hydrolysis.* A 7–8 ml. portion of concentrated sulfuric acid was added to 100 ml. of plasma. The flask was fitted with a reflux condenser to prevent evaporation and put in a boiling-water bath for thirty minutes. The temperature of the plasma in the flask was checked; due to local factors it never exceeded 85° C.

6. *Continuous extraction.* It has been shown by Lieberman *et al.* (16) that continuous extraction is one of the best methods of hydrolysis of the 3β-hydroxy-17-ketosteroids. The continuous extraction apparatus described by Ashley and Murray (17) was modified so that there was continuous stirring of the plasma by a magnetic stirrer. Plasma was brought to pH 0.5 to 0.8 with concentrated sulfuric acid and continuously extracted for forty-eight hours with ether.

D. *Extraction*

In the continuous extraction technique the ether extract was readily available. The other hydrolysates were extracted three times with approximately 1.5 volumes of ether. Each extract was washed twice with 0.1 volume of 10 per cent $NaHCO_3$ and twice with 0.2 volume of distilled water. The washed extract of 100 ml. of plasma was evenly distributed among eight 125-ml. Erlenmeyer flasks and the ether removed with a stream of air.

E. *Florisil-column chromatography*

Columns were prepared as described by Nelson and Samuels (18). Three systems of elution were tried:

Ethyl acetate—ligroin.

Hexane—chloroform—2 per cent methanol in chloroform.

Chloroform—2 per cent methanol in chloroform.

In most cases the columns were finally eluted with 25 per cent methanol in chloroform to check on the effectiveness of previous elutions.

F. *Removal of phenols*

The eluate from the Florisil column which contained the 17-KS was evaporated to dryness. The residue was dissolved in toluene, chloroform, or 2 per cent methanol in chloroform and then washed twice with 0.2 volume of 1 N NaOH and three times with 0.3 volume of distilled water. The pH of the extract at this point should be neutral.

G. *Paper chromatography*

The final dry extract was dissolved in a small amount of methanol-chloroform (1:1 by volume) and transferred to a strip of Whatman No. 2 filter paper. The extract was concentrated on the origin with ethyl acetate-methanol (2:1) according to the method of Bush (19). The paper was placed in a chromatography tank containing the system of solvents, light petroleum-methanol: water (85:15), equilibrated overnight and then chromatographed for approximately four hours.

Reference steroids (DHA and androsterone) were routinely chromatographed alongside the extracts.

H. *Elution from the paper*

The paper strip containing the reference steroids was sprayed with the Zimmermann reagents as described by Bush (13). The areas of the plasma-extract paper strip corresponding to the reference steroids were delimited, cut out and eluted.

I. *Micro-Zimmermann reaction*

The eluate was evaporated to dryness and the residue dissolved in 0.2 ml. of absolute ethanol; 0.2 ml. of 2.5 N KOH in absolute ethanol and 0.2 ml. of 1 per cent *m*-dinitrobenzene were added. The tube was stoppered, shaken and incubated at 25° C. for ninety minutes in the dark. Then 0.6 ml. of distilled water and 0.6 ml. of distilled chloroform were added, the tube was shaken and centrifuged for two minutes. The chloroform layer

was transferred into a microcuvette and read against a reagent blank in a Beckman DU spectrophotometer. Optical density measurements were made at 440, 520, and 600 mμ. Readings were also taken on a DHA and androsterone standard.

RESULTS

Hydrolysis and extraction

The direct extraction of plasma with ether or chloroform did not yield any 17-KS (assay C-1, Table 1). As much as 350 ml. of plasma was extracted with chloroform, fractionated on Florisil columns and chromatographed on paper without any positive results.

The same thing was true after β-glucuronidase hydrolysis (assay C-9,

TABLE 1. YIELD OF 17-KS (DHA AND ANDROSTERONE), USING VARIOUS METHODS OF HYDROLYSIS

Assay No.	Material*	Hydrolysis		Acid added (ml. per 100 ml. plasma)	17-KS (μg./100 ml.)	
		Method	Time		DHA	Andro.
C-1	100 ml. plasma	None	—	—	0	0
C-9	100 ml. plasma	β-glucuronidase	15 hrs.	pH 4.6	0	0
C-10	100 ml. plasma	Hot plate	10 min.	HCl-10 ml.	10	4–5
C-7	100 ml. plasma	Pptn. of proteins, then hot plate	10 min.	HCl	5–8	—
C-22	100 ml. plasma	Water bath	45 min.	H_2SO_4-8 ml.	19	1.8 (?)
C-28	Residue from C-22	Water bath	45 min.	8 ml.-more	8	2 (?)
CR-28	Residue from C-28	Water bath	45 min.	8 ml.-more	0	0
C-23	100 ml. plasma	Water bath	45 min.	H_2SO_4-16 ml.	23.5	2.6
CR-23	Residue from C-23	Water bath	45 min.	8 ml.-more	0	0
C-26	100 ml. plasma	Continuous extr'n.	48 hrs.	H_2SO_4-pH 0.5	42	10
C-27	Residue from C-26	Water bath	45 min.	8 ml.-more	1.7 (?)	0
CR-27	Residue from C-27	Water bath	45 min.	8 ml.-more	0	0
C-30	100 ml. plasma	Continuous extr'n.	48 hrs.	H_2SO_4-pH 0.5	49.5	10.5
CR-30	Residue from C-30	Water bath	45 min.	8 ml.-more	0	0
C-33	50 ml. plasma	Continuous extr'n.	48 hrs.	H_2SO_4-pH 1.	43	8.5
C-34	Residue from C-33	Continuous extr'n.	24 hrs.	—	0	1.0 (?)

* The same stock plasma was used throughout this hydrolysis study.

Table 1). Neither DHA nor androsterone was found after paper chromatography of the extracts. However, when dehydroepiandrosterone glucuronoside[1] was added to plasma, and the mixture incubated with enzyme, we were able to recover 73–81 per cent of the added material as free DHA (Table 2).

[1] The authors wish to thank Dr. Seymour Lieberman who provided the crystalline dehydroepiandrosterone glucuronoside.

TABLE 2. DHA-GLUCURONOSIDE RECOVERY FOLLOWING β-GLUCURONIDASE HYDROLYSIS

Assay No.	Amt. of plasma (ml.)	2 ml. AcOH; 2 ml. AcONa; Penicillin	β-Glucuronidase (ml.)	Added DHA-Glucuronoside as DHA (μg.)	Added DHA (μg.)	DHA Recovery	
						μg.	% of expected amt.
1	15	Yes	4	42.25	0	34.2	81
2	15	Yes	4	84.5	0	61.5	73
3	15	Yes	0	42.25	0	1.0(?)	
4	15	Yes	4	0	57.6	45.5	79.5
5	15	Yes	0	0	57.6	42.0	73
6	15	Yes	4	0	0	Pooled 1.5(?)	
7	15	Yes	4	0	0		
8	15	Yes	4	0	0		

With 10 per cent hydrochloric-acid hydrolysis (assay C-10, Table 1), as well as the method described by West *et al.* (6) in which the proteins were precipitated with ethanol prior to HCl hydrolysis (assay C-7, Table 1), recoveries of 17-KS were low.

The plasma levels of DHA and androsterone were higher after sulfuric-acid hydrolysis. It was found that 16 ml. of H_2SO_4 per 100 ml. of plasma was about the optimal amount of acid for this technique (assays C-22 and C-23, Table 1).

Continuous extraction at pH 1 or 0.5 for forty-eight hours with ether, appeared to be the best method of hydrolysis (assays C-26, 30 and 33, Table 1). A further 24-hour continuous extraction (assay C-34), or a further H_2SO_4 hydrolysis (C-27 and CR-30, Table 1) following the preliminary continuous extraction, did not yield more 17-KS.

TABLE 3. EFFECT OF VARIOUS EXTRACTION PROCEDURES ON THE LEVELS OF PLASMA 17-KS IN A CASE OF CUSHING'S SYNDROME

Treatment before continuous extraction at pH 0.5 with ether for 48 hrs.	Magnetic stirring during continuous extraction	17-KS(μg./100 ml.)	
		DHA*	Andro.*
None	Yes	94.5	76
None	No	58.0	41.0
Butanol extraction	No	91.5	83.5
Ethanol extraction	No	90.0	75.0

* Each figure represents the average of 2 determinations.

TABLE 4. RECOVERY OF 17-KS FOLLOWING CHROMATOGRAPHY ON FLORISIL COLUMNS, USING VARIOUS AMOUNTS OF ELUATE

Compound	Amount (μg.)	% Recovery in fraction*		
		chloroform	2% MeOH in chloroform	25% MeOH in chloroform
		[*50 ml.*]	[*35 ml.*]	[*45 ml.*]
DHA	288	14.7	84.1	0
Androsterone	100	15.3	80.6	0
		[*25 ml.*]	[*25 ml.*]	[*45 ml.*]
DHA	144	5.1	92.3	0
Androsterone	25	6	96.5	0
	50	3.2	95	0
		[*20 ml.*]	[*25 ml.*]	[*45 ml.*]
DHA	144	0	89	5.3
Androsterone	100	0	86.4	7.1
		[*20 ml.*]	[*35 ml.*]	[*45 ml.*]
DHA	144	0	98.8	0
	72	0	92	0
Androsterone	100	0	93.5	0
	50	0	96.5	0

* Each figure is the mean value of 4 determinations.

TABLE 5. RECOVERY OF ESTROGEN FOLLOWING CHROMATOGRAPHY ON FLORISIL COLUMNS

Compound	Amount (μg.)	% Recovery in eluate		
		20 ml. chloroform	35 ml. 2% MeOH in chloroform	45 ml. 25% MeOH in chloroform
Estrone	10	0	102	0.3
		0.2	105	0.2
		0.2	98	2
Estradiol-17β	10	0	101	0
		0	103	1.2
		1	98.5	0
Estriol	10	0	0	88.5
		0	0	90
		0	0	93.5

TABLE 6. RECOVERY OF 17-KS FOLLOWING PHENOLIC FRACTIONATION

Compound	Amount (μg.)	% Recovery
Androsterone	25	91, 97, 95.5, 96.5
Etiocholanolone	29	91, 108, 105, 94
DHA	28.8	97.5, 95.5, 91, 95, 94
Δ^4-Androstenedione	28.6	100, 93, 90, 104
Androstane-3,17-dione	25	93, 100, 101, 102

Trouble may arise during the continuous extraction procedure. The plasma may become very thick and the magnetic stirrer may stop turning, in which case the resulting extraction will be incomplete (Table 3). In order to avoid this difficulty, the plasma should be extracted with butanol or ethanol prior to continuous extraction, the alcoholic extract evaporated to dryness, and the residue taken back into water. The aqueous residue is then brought to pH 1–0.5 and continuously extracted (Table 3).

Column chromatography

The purpose of the Florisil column is to eliminate as much fatty material as possible from the steroid extract. Florisil columns using the system of solvents, chloroform-2 per cent methanol in chloroform, were found to be the most satisfactory. The effect of various amounts of eluate on the recovery of DHA and androsterone from the Florisil columns is shown in Table 4.

TABLE 7. DERIVED OPTICAL DENSITY OF 9.2 μG. OF DHA, USING VARIOUS MODIFICATIONS OF THE ZIMMERMANN REACTION

KOH	Water (ml.)	$CHCl_3$ (ml.)	Derived optical density	Remarks
Aqueous, 5N	—	0.4	.339, .340	Cloudiness
		0.6	.316, .323	Cloudiness
		1.2	.190, .191	Cloudiness
Alcoholic, 2.5 N	0.2	0.6	.408, .382	
	0.4	0.6	.426, .464	
	0.6	0.6	.482, .503	
	0.6	0.8	.375, .366	
	0.6	1.0	.268, .281	
	0.6	1.2	.223, .234	
	1.2	1.2	—	Fading of color

Removal of the phenols

When using the optimal system of elution for DHA and androsterone, certain phenols, such as estrone and estradiol-17β, are also found in the 17-KS eluate (Table 5). For this reason it appeared necessary to wash the phenols out of the eluates. When various 17-KS were dissolved in chloro-

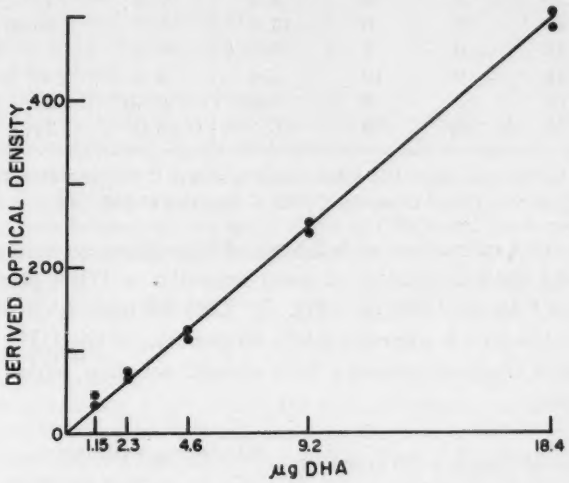

FIG. 1.: Derived optical density of various amounts of dehydroepiandrosterone standard.

$$\left(\text{Derived optical density} = \text{OD at } 520\ \text{m}\mu - \frac{\text{OD } 440\ \text{m}\mu + \text{OD } 600\ \text{m}\mu}{2}\right)$$

form, their recoveries after washing with 1 N NaOH and water were satisfactory (Table 6).

Paper chromatography and elution from paper

As described in the method section, paper chromatography and elution of 17-KS from paper are quantitative procedures. Recoveries of 95 to 103 per cent could be obtained.

Micro-Zimmermann reaction

The present adaptation of the Zimmermann reaction to micro amounts of 17-KS has the advantage of eliminating the interference caused by nonketonic chromogens, as does the Cahen and Salter modification (20) of Holtorff and Koch's method (21).

As shown in Table 7, the addition of 0.6 ml. of water to the test solution

TABLE 8. RECOVERY OF 17-KS THROUGHOUT THE ENTIRE PROCEDURE

Assay No.	Amt. of plasma (ml.)	17-KS added		17-KS found		% Recovery*	
		DHA	Andro.	DHA	Andro.	DHA	Andro.
C-280	25	0	0	—	2.3		
C-281	25	5	0	9.4	2.4	75	
C-282	25	10	0	13.3	2.5	76.5	
C-283	25	0	5	5.7	6.5		82
C-284	25	0	10	5.6	9.6		72
C-285	25	5	5	9.95	6.05	86	73
C-285	25	10	10	13.1	10.0	74.5	76

* Compared to average amount of DHA found in assays C-283 and C-284 and average amount of androsterone found in assays C-280, C-281 and C-282.

followed by a color extraction with 0.6 ml. of chloroform seemed to give the highest derived optical density. A good linearity of DHA standard was found between 1.15 and 18.4 μg. (Fig. 1). Derived optical density[2] of the androsterone standard is approximately 85 per cent of the DHA standard. Δ^4-Androstene-3,17-dione causes a very cloudy reaction which interferes with the reading.

[2] Derived optical density = OD at 520 mμ $- \frac{\text{OD 440 m}\mu + \text{OD 600 m}\mu}{2}$

TABLE 9. DUPLICATION OF RESULTS

	Amt. of plasma used for each determination (ml.)	Plasma 17-KS (μg./100 ml.)	
		DHA	Andro.
Control adult male	40	57.5	12.7
		48.5	9
		46	10.6
		48	7.5
Control adult male	25	30.6	16.2
		30.8	12.8
		28.4	11.5
		35.8	—
Control adult female	35	36.5	19.4
		23.7	17
Cushing's syndrome (adrenal carcinoma)	20	97.0	80.0
		92.0	72.0

TABLE 10. METHOD FOR MEASUREMENT OF DHA AND ANDROSTERONE IN PERIPHERAL VEIN PLASMA OF MAN

1. *Ethanol extraction*
   20 ml. plasma + 100 ml. EtOH: stir, centrifuge, remove EtOH extract.
   Wash protein residue with 50 ml. EtOH, centrifuge, add EtOH to first extract.
   Evaporate EtOH; add 25 ml. water.
2. *Continuous extraction*
   Adjust pH to 0.8–1.0 with H_2SO_4.
   Extract for 24 to 48 hours with ether.
3. *Washing of the extract*
   Reduce ether volume to approximately 150 ml.
   Wash with 10% $NaHCO_3$ (10 ml. × 2) and water (20 ml. × 2).
   Distribute ether equally in two flasks and evaporate to dryness.
4. *Column chromatography*
   Prepare two Florisil columns
   Dissolve each half-extract in exactly 20 ml. of $CHCl_3$ and pour over each column.
   Elute with 35 ml. of 2% MeOH in $CHCl_3$.
5. *Removal of phenols*
   Continue the two eluates and wash with 1 N NaOH (10 ml. × 2) and water (20 ml. × 2).
6. *Paper chromatography*
   Evaporate to dryness.
   Transfer extract onto paper, with DHA and androsterone alongside.
   Chromatograph in Bush light petroleum-methanol:water (85:15) system.
   Elute area of chromatogram corresponding to DHA and androsterone.
7. *Micro-Zimmermann reaction*

Two reagent blanks, 2 DHA standards (9.2 μg.) and 2 androsterone standards (10 μg.) are run with each set of unknowns. The unknowns are read against the reagent blank.

17-KS recovery throughout the entire procedure

Recoveries of DHA and androsterone added to plasma are shown in Table 8. It was possible to recover 72 to 86 per cent of the added 17-KS.

Duplication of results

As shown in Table 9, duplication of results was satisfactory, and a maximum variation of approximately 20 per cent could be expected from duplicate samples.

DISCUSSION

From the data presented, a practical technique for measurement of the 17-ketosteroids, DHA and androsterone, in the plasma of man was devised as outlined in Table 10. One must point out that recoveries of DHA and androsterone were only 72 to 86 per cent, and 20 per cent variation

could be expected in duplicate determinations. Standard urinary 17-KS methods are known to have the same range of recovery and variability and, therefore, the present technique for plasma 17-KS can be considered satisfactory.

This method for plasma 17-KS is satisfactory also from the standpoint of specificity. We have stressed in the introduction the necessity of having the 17-KS well separated from nonspecific compounds which show the Zimmermann reaction. Using the system of solvents, light petroleum-methanol:water (85:15), 17-ketosteroids have smaller R_f values than those of nonspecific compounds which can be visualized following Zimmermann development at the front of the chromatogram. Such nonspecific compounds are present even when the extract for paper chromatography shows only slight amounts of fatty material or none at all. They usually absorb ultraviolet light at 240 mμ and are fluorescent in ultraviolet light. Following chromatography, every paper should be checked on the ultraviolet scanner (22, 23). If a dark stream from the origin to the solvent front be found, the area of the paper corresponding to DHA and androsterone should be eluted and the eluate rechromatographed. This is also true when the nonspecific material at the front is so abundant that it covers the androsterone area of the paper strip.

Another advantage of this technique is that specific 17-ketosteroids are separated and measured. There is some evidence (24) that DHA is produced, or is a metabolite of steroids produced, by the adrenal glands. On the other hand, androsterone and etiocholanolone are known to be the main urinary metabolites of testosterone. The ratio DHA:androsterone might be of interest in various physiologic or pathologic conditions. Androsterone and etiocholanolone, however, are also metabolites of dehydroepiandrosteroone and, therefore, can be of adrenocortical origin.

In 15 normal adult males, the average DHA level was found to be 40.5 μg. per 100 ml. of plasma (range, 29–69 μg.), and that of androsterone 18 μg. (range, 9–39 μg.), giving a ratio of 2.5 (range, 1.2–5.0). The levels in 8 normal adult females were found to vary somewhat with the period of the menstrual cycle but were not significantly different from those found in males (25). Children before puberty had low plasma 17-KS levels. Large individual variations were noted both in males and females, but the number of subjects studied in each group was small. Various clinical studies are in progress and will be reported soon.

SUMMARY

A method has been devised for the measurement of dehydroepiandrosterone (DHA) and androsterone in human plasma. The technique consists of the following steps: 1) ethanol extraction of 25 ml. of plasma; 2)

evaporation of ethanol extract and solution of the residue in water; 3) continuous extraction with ether at pH 0.8 for forty-eight hours; 4) washing of the ether extract with sodium bicarbonate and water; 5) chromatography on Florisil columns; 6) removal of phenols with 1 N sodium hydroxide; 7) paper chromatography and elution of paper areas corresponding to DHA and androsterone; and 8) micro-Zimmermann reaction.

In 15 normal males the average plasma level of DHA was 40.5 μg. and of androsterone, 18 μg. per 100 ml. In 8 normal females, the levels were not significantly different from those in males.

Acknowledgments

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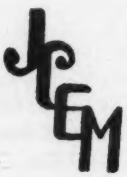

FURTHER EVIDENCE THAT HYPERTHYROIDISM (GRAVES' DISEASE) IS NOT HYPERPITUITARISM: EFFECTS OF TRIIODOTHYRONINE AND SODIUM IODIDE*†

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RECENT work in this laboratory (1, 2) has suggested that Graves' disease is not the consequence of hyperpituitarism but rather is mediated by mechanisms apart from the anterior pituitary. The present study was undertaken to pursue this possibility.

In the initial studies, thyroid and thyrotropin were given to patients with active Graves' disease following testing with I^{131}. Large doses of thyroid failed to suppress the increased I^{131} uptake of the diseased thyroid, though markedly lessening uptake during a remission of the disease following treatment, and in euthyroid control subjects. Thyrotropin failed to increase I^{131} uptake by the thyroid of the patients with active Graves' disease, but caused a sharp increase in the serum precipitable iodine (SPI) level. Control subjects showed sharp increases in both uptake and SPI levels following thyrotropin injections. Morgans and Trotter (3) obtained results similar to those with thyroid, by administering *l*-thyroxine to euthyroid patients and those in a state of remission from hyperthyroidism.

The failure of administered thyroid to suppress I^{131} uptake by the hyperactive thyroid gland of Graves' disease suggested that the normal pituitary-thyroid relationship was deranged in this disorder, and that the disease was not due simply to a surplus of thyroid hormone being maintained by a normally functioning pituitary-thyroid mechanism. The other possibility—that there was hyperactivity of the pituitary because of loss of responsiveness of this gland to thyroid hormone—appeared equally unlikely, in view of the fact that an increase in the SPI level followed the

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administration of thyrotropin, suggesting that the hormonal effect was not lost by dilution in a large pool of excess endogenous thyrotropin.

Objection has arisen to these experiments on the grounds that inorganic and organic iodine-containing substances in desiccated thyroid might have influenced the results; and that the doses of thyroid which were employed might not have been adequate to suppress a hyperfunctioning pituitary. Accordingly, an investigation of the effects of *l*-triiodothyronine (4, 5) was undertaken.[1] In addition, the effects of small doses of iodide were also tested, since iodides cause relief of hyperthyroidism and iodine has been suspected by some as causing inhibition of the action of thyrotropin (6). However, iodide does not affect the release of labeled hormone from the thyroids of rats receiving thyrotropin (7), or from the thyroids of patients treated with iodides and then given the hormone (8).

The results of the present investigation appear to strengthen the conclusion that Graves' disease is not mediated through the anterior pituitary. As an offshoot of these studies, a diagnostic test for hyperthyroidism has been provided.

METHODS

The same clinical, chemical, and radioiodine methods used in the earlier studies in this laboratory were employed. L-triiodothyronine was provided by courtesy of Dr. A. Heming of Smith, Kline and French. At first pills of 35 μg. strength were furnished, but later the potency was reduced to 25 μg. When larger doses were given, the crystalline material was weighed in the laboratory on an air-damped balance, accurate to 0.1 mg. In all tests the material was given by mouth, after breakfast, for each of eight days. The usual daily dose of triiodothyronine was 70–75 micrograms, but doses up to 2 milligrams daily were also used. Before treatment with the drug was begun, the 24-hour I^{131} uptake by the thyroid was determined. A 15-microcurie test dose of I^{131} was given orally, and the radioactivity accumulated by the gland was measured twenty-four hours later by means of a bismuth end-window Geiger tube. In 6 instances, the 72-hour SPI level (9) was determined by a modification of the original method, permitting the measurement to be made without need for additional I^{131}. This was rendered feasible through the use of a heavily shielded well-type scintillation counter for liquid counting (10). On the morning of the eighth day of triiodothyronine dosage, residual radioactivity in the thyroid was measured and another 15-μc. dose of I^{131} was administered by mouth. Uptake by the thyroid was then determined twenty-four hours later, allowing for residual activity.

Thyrotropin was provided by the kindness of Dr. E. Hays of Armour and Co., in ampules of 10 Armour units (30 mg.) each of lyophilized material. The powder was dissolved in 5 cc. of saline and injected intramuscularly (10 units daily for three days). A 24-hour uptake was determined before the thyrotropin was administered, using a 15-μc. I^{131} oral test dose. On the third day of thyrotropin injection, residual radioactivity within the thyroid was measured and 15 μc. of I^{131} again given by mouth. The 24-hour uptake was determined the next day, due allowance being made for the residual activity

[1] A preliminary note concerning these results has appeared (2).

in the gland. Blood for determination of SPI was drawn just before, and seventy-two hours after, the start of thyrotropin therapy.

Sodium iodide, C. P., was dissolved in distilled water and given, when employed, throughout the experiment in daily oral doses of 1.4, 2.8, 5.6, and 11.2 mg. after dinner. The 24-hour I^{131} uptake and SPI level were determined before, and during iodide therapy. The status of the patients' hyperthyroidism was also checked clinically. The improvement effected by iodide therapy occurred within one to two weeks of the start of treatment in all patients receiving 5.6 and 11.2 mg. daily, and in most of the others. When improvement was evident, thyrotropin was administered in a dosage of 10 units intramuscularly daily for three days. The SPI level and 24-hour I^{131} uptake were then determined as previously indicated.

RESULTS

I. *Triiodothyronine*

A. *Overt hyperthyroidism.* Nineteen patients with unquestioned active Graves' disease receiving the 0.07–0.75 mg. dose of triiodothyronine described showed an average 24-hour I^{131} uptake before treatment of 56 per cent, and at the end of treatment, 62 per cent (Table 1). No uptake less than 36 per cent was noted following therapy.

TABLE 1. EFFECT OF TRIIODOTHYRONINE ON 24-HOUR I^{131} UPTAKE BY THE THYROID

Patient group	Triiodothyronine, daily dose (mg.)	No. of patients	Thyroidal I^{131} uptake (%)			
			Before		After	
			Avg.	Range	Avg.	Range
A. Active hyperthyroidism	0.075	19	56	15–72	62	36–79
	2.0*	7	71	49–96	63	36–75
B. Euthyroid, with eye signs only	0.075	8	37	10–68	36	10–64
	0.15	1		20		17
	0.5	3		10, 24, 38		3, 35, 54
	1.0	2		33, 49		20, 30
C. Inactive hyperthyroidism	0.075	6	26	16–40	9	3–20
D. Control subjects	0.075	30	35	12–64	11	1–36†

* Data taken from previously published work (2); 1 patient received 1 mg. daily.

† Only 1 subject with an uptake in excess of 20 per cent; this decreased to 1 per cent, with 0.15 mg. triiodothyronine daily.

In 6 patients, a dose of 2 mg. of triiodothyronine daily was given for six days, and in 1 additional patient, 1 mg. daily for the same length of

time. The intensity of the flare-up of symptoms necessitated cessation of therapy at this point. Again no significant suppression of thyroidal I^{131} uptake occurred (Table 1). The uptake averaged 71 per cent before, and 63 per cent at the end of the course of treatment.[1]

B. *Euthyroid patients with early eye signs of Graves' disease.* Ten patients were observed with a recent history of onset of eye signs of Graves' disease but with no evidence of hyperthyroidism (Fig. 1). The salient clinical and laboratory features of this group have been listed elsewhere (11). In Table 1 are recorded the responses to small and large doses of triiodothyronine. Although a small, but probably significant depression of uptake occurred in the 2 patients given daily doses of 1 mg. of triiodothyronine, daily doses of 75, 150 and 500 μg. caused no significant change. With the 75-μg. dose, average I^{131} uptake before treatment was 37 per cent, and at the end, 36 per cent.

FIG. 1. Patient A.M., female, aged 45, showing stare and widened ocular fissures, characteristic early eye signs of Graves' disease, despite the euthyroidism. BMR+3 per cent; thyroidal 24-hour I^{131} uptake, 44 per cent; SPI level, 8.1 μg./100 ml.; fasting serum cholesterol level, 260 mg./100 ml.

C. *Sustained remission of Graves' disease.* Six patients in whom Graves' disease was brought into a state of remission by means of definitive therapy and in whom the remission was sustained for one or more years, were given triiodothyronine (Table 1). The 24-hour I^{131} uptake before triiodothyronine administration averaged 26 per cent; at the end it was 9 per cent. In all but 1 instance uptake fell to less than 20 per cent following the use of the drug.

D. *Euthyroid control subjects.* Patients with no evidence of thyroid dysfunction were given 70 or 75 μg. of triiodothyronine. The 24-hour thyroidal uptake of I^{131} was determined before and after treatment. Uptake prior to therapy averaged 35 per cent, and at the end, 11 per cent. In only 1 instance did uptake fail to decrease to less than 20 per cent following administration of the drug. In this patient, an uptake of 1 per cent was obtained when the dose of triiodothyronine was increased to 150 μg. daily for eight days.

TABLE 2. EFFECTS OF THYROTROPIN UPON 24-HOUR THYROIDAL I^{131} UPTAKE BY THE THYROID AND UPON SPI LEVEL

Patient group	No. of patients	Average thyroidal I^{131} uptake (%)		Average SPI (μg./100 ml.)	
		Before	After	Before	After
A. Active hyperthyroidism*	22	59	58	12.5	15.1
B. Euthyroid, with eye signs only	8	36	43	6.7	11.4
		Range 10–54	18–58	4.5–9.5	7.1–16.8
C. Inactive hyperthyroidism*	14	31	38	6.0	6.8
D. Control subjects*	7	25	47	6.1	10.4

* Data taken from previously reported work (1).

II. *Thyrotropin*

The effects of thyrotropin in active Graves' disease, Graves' disease in a state of remission following therapy, and in euthyroid control subjects have been reported from this laboratory (1). The results are summarized in Table 2 for purposes of comparison with the effect of thyrotropin upon the euthyroid patients with early eye signs of Graves' disease (Group B

TABLE 3. EFFECT UPON 24-HOUR THYROIDAL I^{131} UPTAKE AND UPON SPI LEVEL OF SMALL DOSES OF SODIUM IODIDE AND THYROTROPIN IN GRAVES' DISEASE

No. of patients	Daily dose of NaI (mg.)	Average thyroidal I^{131} uptake (%)			Average SPI (μg./100 ml.)		
		Before treatment	After NaI	After NaI & thyrotropin	Before treatment	After NaI	After NaI & thyrotropin
1	0.7	66	3	68	10.0	9.5	17.3
1	1.4	53	23	23	10.0	7.0	11.9
5	2.8	60	13	23	10.6	7.5	14.2
6	5.6	63	17	32	11.2	8.8	13.4
6	11.2	60	16	20	11.9	8.9	11.6

of the present study). The results in Group B revealed little increase in I^{131} uptake after thyrotropin injections, but a distinctly significant elevation in the level of SPI.

III. *Sodium iodide*

The effects of sodium iodide administration in active Graves' disease upon 24-hour thyroidal I^{131} uptake and upon the level of SPI is shown in Table 3, together with the respective responses to thyrotropin. The markedly reduced thyroidal uptake of I^{131} which resulted from iodide therapy was increased only to a limited extent when thyrotropin was injected, whereas the concentration of SPI, lowered practically to normal by iodide therapy, was increased sharply and attained levels higher than those originally present.

DISCUSSION AND CONCLUSION

The failure of triiodothyronine to depress thyroidal I^{131} uptake in Graves' disease provides indisputable evidence that the normal reciprocal pituitary-thyroid relationship is disrupted. The 2-mg. daily doses of triiodothyronine were the approximate equivalent of 2.6 to 3.2 Gm. of thyroid daily, and caused enough clinical exacerbation of the hyperthyroidism to necessitate stopping treatment after six days. The concept is thus untenable that hyperthyroidism is due to a pituitary "thermostat" set to maintain too high a blood level of thyroid hormone but which nevertheless functions normally at this new level. The failure of the pituitary to be suppressed by either thyroid or triiodothyronine also makes untenable the alternative possibility, that the pituitary becomes hyperactive because the thyroid in Graves' disease secretes an abnormal thyroid hormone which cannot suppress the pituitary. No abnormal secretion has been found in hyperthyroidism (12, 13).

However, the possibility remains that hyperthyroidism might be caused by a change in the pituitary so that it no longer is suppressed by the normal thyroid hormone and thus becomes hyperactive. The present evidence, although admittedly indirect, fails to bear out such a concept. The fact that administered thyrotropin induces release of thyroid secretion indicates that there is no loss of effect by dilution in a large pool of excess endogenous thyrotropin. The different effects of thyrotropin upon uptake, which was little influenced by the thyrotropin therapy, and upon release, which was sharply stimulated, also are inconsistent with a disease caused by thyrotropic stimulation.

The suggestion of an extra-pituitary orgin is emphasized when the responses of the euthyroid patients with early signs of Graves' disease (Group B) are examined (11). The results of triiodothyronine administration

revealed the normal pituitary-thyroid relationship to be disrupted, as in Graves' disease with overt hyperthyroidism. Furthermore, the low or normal I^{131} uptakes in this group were not significantly increased by thyrotropin injection, a finding which cannot be attributed to the fact that the trapping capacity of the thyroid was already at a maximum and hence incapable of further stimulation. Thus, initial uptakes for the group averaged only 36 per cent and, in several instances, the uptake was less than 20 per cent. The release of thyroid hormone, on the other hand responded as in normal subjects. Moreover, were an excess of endogenous thyrotropin the cause of the disorder in these patients, release of thyroid hormone probably would have been occurring at an increased rate, since this is considered to be the first response to thyrotropin of the normal thyroid. Nevertheless, the 72-hour SPI^{131} levels of 4 of the 6 patients so tested were normal; none of the patients was overtly hyperthyroid and the SPI levels of 7 of the 10 patients were less than 7.5 gamma per 100 ml. Thus the euthyroid status of these patients is difficult to reconcile with the view that an excess of thyrotropin causes Graves' disease.

It is possible that these patients had had Graves' disease and that a spontaneous remission had occurred by the time they were seen. This seems unlikely in view of the brief duration of symptoms. Even so, the foregoing conclusions seem valid. It is also possible that these patients did not have Graves' disease, since none of the patients has as yet become hyperthyroid.[2] This is unlikely, however, since their clinical appearance is so characteristic of Graves' disease (Fig. 1). Moreover, in 2 patients not included in the series, severe hyperthyroidism developed within one to two years from the time of onset of the eye signs.

The final argument against a hyperpituitary concept of Graves' disease is presented by the results of the iodide experiment. A striking amelioration was caused by iodide administration. The recovery, occurring in the presence of an excess of thyrotropin, would necessarily imply interference with the effect of thyrotropin. Nevertheless, when small doses of thyrotropin were administered, the release of thyroid hormone was great enough to suggest an increased sensitivity to the hormone. The concurrent relative unresponsiveness of the I^{131} uptake to thyrotropin was similar to that observed in untreated hyperthyroidism. However, flooding of the trapping mechanism by the large amount of iodide administered could account for this poor response.

The evidence, then, suggests that there is no excess of thyrotropin present in Graves' disease and that hyperpituitarism is not the causative factor. That the thyroid has an intrinsic mechanism, not under pituitary

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[2] Overt thyrotoxicosis has developed in 2 patients since this paper was presented (11).

control, for trapping iodine and regulating the extent of this trapping during iodine lack, has been recently demonstrated in the hypophysectomized rat (14). Derangement of such a mechanism could result in a disorder such as Graves' disease, as could a derangement of other extrapituitary processes which, in turn, might control this mechanism or those as yet undetermined. As a corollary, the study suggests that iodides do not act in Graves' disease by inhibiting the effect of thyrotropin, but rather act directly on the overactive thyroid cell, partly at least, by inhibiting the release of thyroid hormone (15).

Finally, the described use of triiodothyronine was found to have considerable diagnostic significance, since failure of suppression of thyroidal uptake is indicative of hyperthyroidism. Further data have been published elsewhere (16). Greer recently used thyroid therapy in the same way and obtained similar favorable results (14).

SUMMARY

1. The effects of triiodothyronine and of thyrotropin have been observed in patients with active Graves' disease, in euthyroid patients with the eye signs of the disease, in patients in a state of sustained remission from the disease, and in euthyroid control subjects.

2. The effect of thyrotropin during a remission induced by short-term treatment with sodium iodide given in small doses is also presented.

3. The evidence suggests the following conclusions: 1) that Graves' disease is not a disorder caused by hyperpituitarism, 2) that iodides act directly upon the thyroid cell and not by interference with the action of thyrotropin, and 3) that the difference in the response to triiodothyronine between patients with active Graves' disease and those with other related conditions provides a useful diagnostic test.

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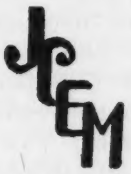

NATURE AND TRANSPORT OF THE IODINATED SUBSTANCES OF THE BLOOD OF NORMAL SUBJECTS AND OF PATIENTS WITH THYROID DISEASE*

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FOR many years various observers, notably Plummer (1), have entertained the attractive idea that abnormal compounds of iodine might account for some of the features of Graves' disease and of the thyrotoxicosis of nodular goiter. The term "dysthyroidism" has been used to convey this idea. Thyroxine is well known to be the dominant iodinated component of the blood of the normal subject (2, 3, 4). Recently 3-5-3'-triiodothyronine has also been demonstrated in the peripheral blood of normal subjects (5). However, no other iodinated compound has been identified in the blood of man, except after heavy radiation.

The iodinated organic components of the blood are closely associated with plasma protein. They are precipitated with the protein and may readily be extracted from plasma with butanol. In addition, blood contains a small amount of inorganic iodide. Gordon, Gross, O'Connor and Pitt-Rivers (6) fractionated the proteins of serum by the method of paper electrophoresis and demonstrated not only that thyroxine labeled *in vivo* by the administration of I^{131} is associated with protein possessing the mobility of alpha-globulin, but that labeled thyroxine added to serum *in vitro* also becomes associated with this same serum protein component. Also Larson, Deiss and Albright (7, 8) found that thyroxine is bound to a serum protein which appears in the electrophoretogram just ahead of the alpha-2 globulin (inter-alpha zone). This protein has been further characterized by Robbins *et al.* (9, 10, 11). Schmid (12) found that the greatest concentration of protein-bound iodine was in an alpha-glycoprotein fraction.

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In the sera of patients who have been given radioactive iodine, a small amount of I^{131} is closely associated with albumin. Deiss *et al.* (8) found this complex to be present early after therapy, and observed that it disappeared more rapidly than did the I^{131} from the inter-alpha zone. They postulated an iodide-albumin bond, but did not report the fraction of the administered dose of I^{131} which was found in the I^{131}-albumin complex. Horst and Roesler (13) found that when I^{131} was added to serum, 6 per cent was bound by albumin and was not extractable with butanol. Smaller amounts were bound by other protein fractions, but highly variable amounts were bound in the albumin zone and in the inter-alpha zone when I^{131} was given to patients with thyrotoxicosis and cancer of the thyroid gland. Robbins and Rall (14) found that the ratio of labeled iodine in the inter-alpha zone to that in the albumin was 85:15 in 6 patients who had been treated with I^{131}. Horst and Roesler (13) confirmed these findings, and observed that this ratio remained constant over a twelve-day period. When serum from patients treated with I^{131} was extracted with butanol and this extract was added to fresh sera, the radioactivity reappeared in the same two positions on the electrophoretogram. Larson, Deiss and Albright (15) separated radioactive fractions of labeled plasma by electrophoresis on a starch block and showed that the radioactivity associated with the alpha-globulin zone was wholly extractable into butanol. They failed to find, however, that the radioactivity associated with the albumin was butanol-soluble.

According to Deiss, Albright and Larson (16), triiodothyronine is associated with the same protein as is thyroxine and can be displaced by thyroxine. Further, these authors found that labeled triiodothyronine in blood does not give a sharp band on the electrophoretogram; they thought this was due to dissociation of the triiodothyronine-protein complex, and that there was no specific binding by other serum proteins.

Thyroglobulin differs from the protein which binds thyroxine in its salting-out characteristics and in its sedimentation rate in the ultracentrifuge, but cannot be separated from it by electrophoresis at pH 8.6 (14). The presence of thyroglobulin in the sera of patients treated with high doses of I^{131} cannot therefore be demonstrated directly by electrophoretic methods.

In the present paper, a comparison has been made of the binding properties of the serum proteins for thyroxine, triiodothyronine and iodide. At the same time, the iodinated compounds in the sera of hyperthyroid and clinically euthyroid subjects have been examined by chromatographic methods, in an attempt to detect abnormal constituents in the sera of hyperthyroid patients.

MATERIAL AND METHODS

Paper electrophoresis was performed at pH 8.6 and ionic strength 0.05, using a veronal buffer (13). A sample of serum (0.01 ml.) was placed on a line on horizontally suspended Whatman #1 filter paper. After a potential of 110 volts had been applied for fifteen to sixteen hours, the paper strips were dried vertically, stained for protein with bromphenol blue and rinsed in 0.5 per cent acetic acid and water.

The electrophoretograms were cut according to their protein pattern or into 1-cm. segments. Radioactivity was then measured either in a deep-well scintillation counter, or by moving the paper at constant speed before a shielded Geiger-Mueller tube; in some cases the strips were autoradiographed on x-ray film. In some experiments, the relative protein concentrations were measured quantitatively by eluting the bromphenol blue dye in 0.01 N sodium hydroxide and determining optical density in a Coleman colorimeter.

Serum for the electrophoretic studies was obtained from 16 patients treated with I^{131}. Two cardiac patients with normal thyroid glands, 2 patients with toxic nodular goiter, 10 with Graves' disease, and 2 with thyroid carcinoma were represented in this group. Serum was also obtained from 2 myxedematous patients given I^{131}-labeled thyroxine intravenously, and from 1 patient with adrenal insufficiency given chromatographically pure I^{131}-labeled triiodothyronine[1] intravenously. In addition, I^{131}-labeled thyroxine[1] was added *in vitro* to serum from 2 normal subjects, and from 4 patients with nontoxic nodular goiter. I^{131}-labeled triiodothyronine was added *in vitro* to normal serum.

Chromatographic examination on paper of I^{131}-labeled compounds in serum was made on *n*-butanol extracts, run either in one dimension in butanol-dioxane-ammonia (Gross, Leblond, Franklin and Quastel (17)) or butanol ammonia, or in two dimensions in butanol-acetic acid and butanol-dioxane-ammonia (17).

The known compounds thyroxine, triiodothyronine and iodide, were either located by autoradiography (17) or by adding carriers to the serum butanol extracts, developing by a phenol reagent and counting the spots thus revealed in a deep-well scintillation counter.

Serum from 27 patients was studied. In many cases serial samples were obtained over the course of the first six days after administration of I^{131}. Many of the studies were made after administration of therapeutic doses of from 5.5 to 35 millicuries of I^{131} for thyrotoxicosis, or after ablation of

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[1] The labeled triiodothyronine and thyroxine were supplied by Abbott Laboratories. Just prior to use each was chromatographed on paper in butanol-dioxane-ammonia and eluted with a mixture of 75 parts of ethanol and 25 parts of ammonia.

the thyroid gland in the treatment of angina pectoris. Other studies were performed after tracer doses of I^{131} ranging from 50 to 400 μc. Seventeen patients had thyrotoxicosis, the diagnosis being confirmed by measurements of basal metabolic rate, serum concentration of protein-bound iodine, and thyroidal I^{131} uptake. Of these cases of thyrotoxicosis, 4 were classified as toxic nodular goiter, and the rest as Graves' disease. Three patients were suspected of having thyrotoxicosis, but objective laboratory evidence failed to confirm the diagnosis. The remaining 10 subjects were euthyroid, but 2 had nodular goiter.

RESULTS

I. *Electrophoretic experiments*

A. *Iodide.* Inorganic iodide migrated rapidly toward the anode and after several hours was well ahead of the leading serum protein (albumin). When carrier-free I^{131} was added to serum for twenty-four hours *in vitro*,

TABLE 1

	Total bound I^{131} in	
	Albumin (% total I^{131})	α-Globulins (% total I^{131})
I^{131}-labeled thyroxine in pure bovine serum albumin*	29.1	—
Same+normal human serum	16.3	65.8
Same+normal human serum	13.3	42.4
Same+normal human serum	8.7	56.5

* Concentration = 50 mg. per ml.

a small fraction, less than 2 per cent, moved with the gamma globulin and albumin components. When carrier iodide (KI) was added to this serum, the protein-bound radioactivity decreased.

B. *Thyroxine.* Thyroxine alone migrates slowly in an electrical field toward the anode (16). When labeled thyroxine was added to pure bovine serum albumin[2] under the conditions of these experiments, it was bound by, and migrated with the albumin. In order to determine the stability of this albumin-thyroxine complex, the foregoing solution was diluted 1:1 with normal human serum. The quantity of labeled thyroxine which was bound to the albumin was measured before and after the addition of normal serum. Upon addition of serum the largest proportion of the thyroxine appeared

[2] Obtained from Armour Laboratories. Bovine serum albumin was dissolved in barbitol buffer to give a concentration of approximately 50 mg. per ml.

in the inter-alpha area of the electrophoretogram, and the proportion bound to albumin decreased (Table 1).

When I^{131}-labeled thyroxine was added to normal serum the predominant radioactivity appeared just ahead of the alpha-2 globulin band. A smaller amount traveled with the albumin. Likewise, serum taken from patients who had received therapeutic doses of I^{131} showed radioactivity associated with protein in the inter-alpha and albumin portions of the electrophoretogram. No difference was noted between sera from normal subjects and those from persons with hyperthyroidism or myxedema. Serum samples obtained from 2 myxedematous patients ten minutes after injections of I^{131}-labeled thyroxine again showed radioactivity in both the inter-alpha and albumin zones.

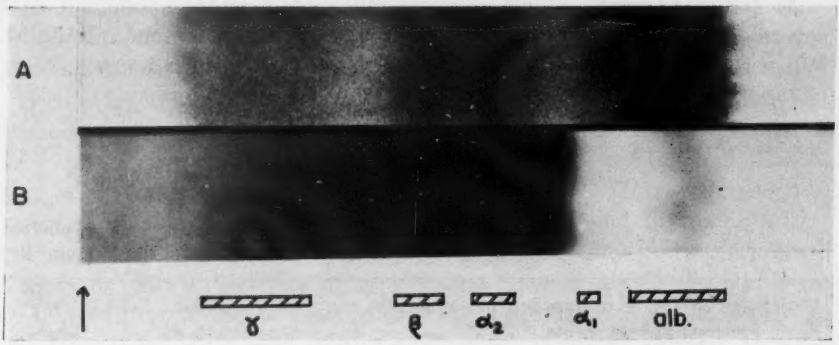

FIG. 1. The upper figure (A) is a paper electrophoretogram stained for protein; the lower figure (B) is an autoradiograph of A. I^{131}-labeled triiodothyronine was added to serum to a concentration of 1.1 μg. per 100 ml., and 0.02 ml. of serum was placed on the paper strip. The locations of albumin (alb.) and the globulins (α, α_2, β and γ) are indicated. Note the preponderant appearance of the labeled compound in the alpha-2 and inter-alpha zones, and also its appearance in other protein bands. The arrow indicates the origin.

The radioactive material was eluted with ethanol-ammonia (75:25) from the two zones in the electrophoretogram of the serum of a hyperthyroid patient forty-eight hours after I^{131} therapy. Analysis of the eluates showed that the predominant labeled compound in both zones was thyroxine.

C. *Triiodothyronine.* I^{131}-labeled triiodothyronine alone does not migrate in an electric field (16). In the present experiments it was shown that when triiodothyronine was added to bovine albumin, it was bound by, and migrated with the albumin. Unlike thyroxine, there was incomplete binding during migration as indicated by smearing on the autoradiograph. When added to serum, I^{131}-labeled triiodothyronine showed binding with all the

globulins, but with a peak concentration in the inter-alpha zone. In addition, a small amount of binding was consistently demonstrated with the albumin fraction (Fig. 1).

Serum obtained from a patient one hour after an intravenous dose of 10 μg. of triiodothyronine labeled with 170 μc. of I^{131} again showed binding with the globulins, but the largest portion of the hormone was found in the inter-alpha and the alpha-2 globulin zones.

II. *Chromatographic experiments*

In all the sera examined by paper chromatography, only 3 compounds were identified (apart from artifacts well recognized by many workers which appear in the alkaline solvents at the solvent front), namely: iodide, thyroxine and triiodothyronine. No triiodothyronine was found in 1 patient with Graves' disease, 1 with a nontoxic nodular goiter and 1 with a normal

TABLE 2

Case	Diagnosis	Time after I^{131} (hrs.)	Dose I^{131}	Labeled triiodothyronine (% of labeled thyroxine)
1	Normal	72	400 μc.	8.7
2	Normal (cardiac)	48	10 mc.	19.2
3	Nontoxic nodular goiter	66	50 μc.	17.7
4	Toxic nodular goiter	48	25 mc.	7.6
5	Graves' disease	48	10 mc.	11.1
		72		4.2
6	Graves' disease	24	6.6 mc.	7.5

thyroid gland. It was noticed, although no quantitative measurements were made, that triiodothyronine seemed to disappear more rapidly in hyperthyroid patients than in euthyroid patients. The sera of 6 patients drawn between twenty-four and seventy-two hours after administration of I^{131}, either in tracer or therapeutic doses, were examined for the relative concentrations of labeled triiodothyronine and thyroxine. Two of the patients had Graves' disease, 1 nontoxic nodular goiter, 1 toxic nodular goiter, and 2 had normal thyroid glands. The results appear in Table 2. Triiodothyronine and thyroxine were observed in all samples.

COMMENT

There is minimal binding of iodide by serum protein, as can be demonstrated electrophoretically. This type of binding may be quite different from that postulated by Danowski (18) to account for the precipitable

iodide observed in the serum of patients who have been treated for a long time with large doses of iodide.

It has been confirmed that thyroxine is transported in the serum, mainly in conjunction with protein which has electrophoretic mobility between alpha-1 and alpha-2 globulin. In addition, a small fraction of serum thyroxine is bound to albumin. The inter-alpha protein-thyroxine bond appears to be one of greater stability. There are no evident qualitative differences in the transport of thyroxine between thyrotoxic patients and normal subjects, and the myxedematous patient is capable of transporting thyroxine normally if it is made available. At present there is no evidence that the availability of a specific protein constitutes a means whereby the body regulates the transport and supply of thyroid hormone to the periphery.

The serum triiodothyronine-protein bond appears to be less discriminatory than that of thyroxine, since triiodothyronine is found in all the electrophoretically distinguishable protein bands. This, however, may be the result of overloading in *in vitro* experiments, and it must be emphasized that the absolute amount of triiodothyronine relative to thyroxine is not known.

Chromatographic studies on hyperthyroid and euthyroid patients failed to reveal any abnormal component in the sera of the former group. The evidence presented does not disprove that some abnormal constituent may exist but it is highly unlikely that, if present, it contains iodine or is present in more than minute amounts. The possibility remains, however, that serum from patients with Graves' disease contains a non-iodinated constituent not present in the serum of euthyroid patients. This is beyond the scope of the present discussion.

CONCLUSIONS

1. It is confirmed that thyroxine is transported in the plasma largely by protein with an electrophoretic mobility between the alpha-1 and alpha-2 globulins, and to a lesser extent by the albumin fraction. The inter-alpha bond is stronger.

2. Triiodothyronine *in vitro* becomes associated with the electrophoretically distinguishable plasma proteins, and has a peak concentration similar to that of thyroxine in the inter-alpha globulin zone.

3. No I^{131}-labeled substances other than iodide, thyroxine and triiodothyronine were found in the sera of 27 patients who received I^{131}. Thirteen of these patients had Graves' disease, 4 had toxic nodular goiter, 2 had nontoxic goiter, and 8 had normal thyroid glands.

4. These studies furnish no evidence that there are qualitative differences between normal subjects and those with various thyroid disorders

either in the iodinated components of the blood, or the mechanisms of their transport.

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A NOTE ON THE IODINATED CONSTITUENTS OF NORMAL THYROIDS AND OF EXOPHTHALMIC GOITERS*

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MANY years ago Plummer (1) suggested that there may be a qualitative abnormality in thyroid function in patients with exophthalmic goiter. However, comparison of normal thyroid tissue with that of untreated exophthalmic goiters (2) led Gutman and co-workers (3) to suggest that the thyroid in untreated thyrotoxicosis is characterized by a lower iodine concentration and proportionately less thyroxine and more diiodotyrosine than normal tissue. The fractionation procedure of Gutman and co-workers for separating iodinated compounds of the thyroid was based on differential solubility and resulted in the resolution of thyroidal iodine into three main fractions: iodide, diiodotyrosine and thyroxine. Subsequent work, however, has shown that each of these fractions contains additional iodinated compounds. Because fractionation procedures for thyroid tissue have in recent years been greatly improved through the use of radioiodine and chromatography, we thought it was worth while to reinvestigate the chemical composition of thyroid tissue in untreated exophthalmic goiter.

MATERIALS AND METHODS

Four patients with mild untreated exophthalmic goiter were given orally 300 to 600 microcuries of I^{131}. After an interval, which varied in each instance ($5\frac{1}{2}$, $7\frac{1}{4}$, 41 and 93 hours after administration of radioiodine) a biopsy specimen of the thyroid was obtained. After biopsy, the patients were treated with Lugol's solution, 10 drops three times a day for seven or ten days. Subtotal thyroidectomy was then carried out, and a second

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sample of tissue was secured. For comparison, 5 euthyroid patients who were to undergo cervical exploration for thyroid tumors (4 cases) or parathyroid adenoma (1 case) were also given orally 300 to 600 microcuries of I^{131}. At time intervals after administration of I^{131} which were roughly the same as in the group with Graves' disease, these patients underwent their scheduled explorations during which a sample of thyroid tissue was obtained for analysis.

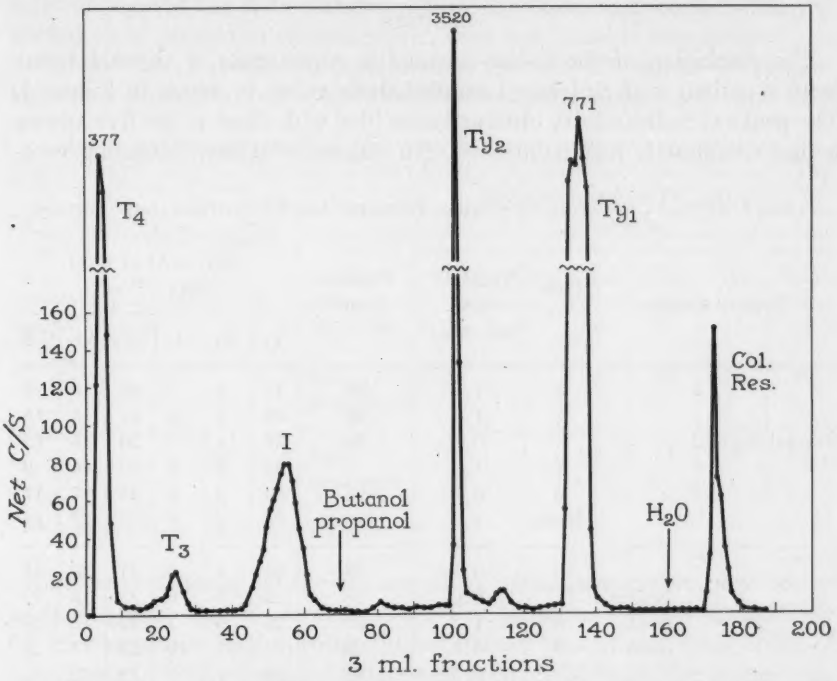

FIG. 1. Effluent diagram (net counts per second) of trypsin hydrolysate of untreated exophthalmic goiter (Case 7) from patient who received I^{131} seven and a quarter hours before biopsy.

T_4 = Thyroxine	Ty_2 = Diiodotyrosine
T_3 = Triiodothyronine	Ty_1 = Monoiodotyrosine
I = Iodide	Col. Res. = Column residual

The tissue obtained from these 9 patients was studied histologically and chemically. Total I^{127} and I^{131} and trichloroacetic acid precipitable I^{127} and I^{131} were determined on each sample. Resolution of the iodine compounds of the tissue was carried out by the method of Braasch and co-workers (4). The tissue was homogenized, hydrolyzed with trypsin, and fractionated by kieselguhr-column chromatography. This method allows recognition

of compounds making up as little as 3 per cent of the total radioiodine content of any sample of tissue. The recovery by this method of resolution is as follows: thyroxine, 72 per cent; triiodothyronine, 84 per cent; iodide, 97 per cent; diiodotyrosine, 91 per cent; and monoiodotyrosine, 90 per cent. I^{127} was determined by the method of Astwood and Bissell (5) and I^{131} by means of a well-type sodium iodide (Tl) crystal scintillation counter connected to a suitable scaling circuit.

RESULTS

The resolution of the iodine-containing compounds of thyroid tissue from a patient with untreated exophthalmic goiter is shown in Figure 1. The peaks of radioactivity obtained coincided with those of the five known iodine compounds just mentioned. No unknown iodine-containing com-

TABLE 1. I^{127} DATA RELATIVE TO NORMAL THYROIDS AND TO EXOPHTHALMIC GOITERS

Type of tissue		Case No.	Total I^{127} conc. (μg./mg.)*	Protein-bound I^{127} (%)*	Per cent† of total peak I^{127} as:					
					T_4	T_3	I	Ty_2	Ty_1	C.R.
Normal thyroid		1	1.3	98	34	4	5	32	19	8
		2	1.0	98	29	6	8	31	15	15
		3	0.6	98	36	14	5	20	16	10
		4	1.2	—	41	8	6	20	16	9
		5	0.7	95	34	9	8	23	17	11
		Mean	*1.0*	*97*	*35*	*8*	*6*	*25*	*17*	*11*
Exophthalmic goiter‡	Before treatment	6	0.5	98	31	4	6	31	20	11
		7	0.6	98	35	9	6	25	15	12
		8	1.2	95	25	5	7	32	15	18
		9	0.3	89	24	11	10	25	17	15
		Mean	*0.7*	*95*	*29*	*7*	*7*	*28*	*17*	*14*
	After treatment	6	0.7	81	27	5	20	24	15	11
		7	0.7	71	23	4	33	18	14	10
		8	0.9	89	25	5	9	26	13	23
		9	0.3	32	8	4	70	8	7	6
		Mean	*0.7*	*68*	*21*	*5*	*33*	*19*	*12*	*13*

Abbreviations: T_4 = Thyroxine; T_3 = Triiodothyronine; I = Iodide; Ty_2 = Diiodotyrosine; Ty_1 = Monoiodotyrosine; C.R. = Column residual

* Each figure in this column is usually an average of several determinations.

† Each figure in this part of table is an average of two duplicate values.

‡ Each case was studied both before treatment and after seven or ten days of treatment with Lugol's solution.

pound was evident in any case. If present, it must have been hidden within one of the peaks of the five known compounds, or it must have been transformed to a known compound. That the former possibility is unlikely is suggested by the results of rechromatographing a radioactive sample from each peak area on paper. These procedures resulted in the demonstration of essentially homogeneous peaks. Chromatograms of normal thyroid tissue or of exophthalmic goiters after treatment with Lugol's solution showed the same pattern. Diiodothyronine and iodohistidine, reported to be present in thyroid tissue, were not found in this study.

Tables 1 and 2 contain data relative to the total I^{127} and to the proportions and concentrations of the five iodine-bearing compounds. The total

TABLE 2. CONCENTRATION OF I^{127} IN FRACTIONS OF THYROID HYDROLYSATES

Type of tissue		Mean micrograms I^{127}/100 mg. tissue as:					
		T_4*	T_3	I	Ty_2	Ty_1	C.R.
Normal thyroid		35	8	6	25	17	11
Exophthalmic goiter	Before treatment	20	5	5	20	12	10
	After treatment	15	4	23	13	8	9

* Abbreviations: same as in Table 1.

iodine concentration of the untreated exophthalmic goiters was somewhat less than that of normal tissue. The proportion of I^{127} precipitable by trichloroacetic acid (protein-bound iodine) was of the same order of magnitude in both untreated goiters and normal thyroids. The proportionate distribution of the five iodine compounds was essentially the same in the untreated exophthalmic goiters and in normal tissue. Comparison of the goitrous glands before and after administration of Lugol's solution showed little change in total I^{127}, a decrease in protein-bound I^{127}, and an absolute and relative increase in the proportion and concentration of iodide.

Table 3 contains radioiodine data relative to the study. It may be noted that the proportionate increase of I^{131} in the thyroxine fraction appeared to occur more rapidly in the untreated exophthalmic goiters than in normal tissue. The specific activity data also support the idea that the turnover of thyroxine was more rapid in the goitrous glands than in normal glands.

TABLE 3. I^{131} DATA RELATIVE TO NORMAL THYROIDS AND TO EXOPHTHALMIC GOITERS

Type of tissue		Case No.	Interval from I^{131} dose to biopsy (hrs.)	Per cent* of total I^{131} as:						Specific activity** of iodine in:						S.A. ratio† tyrosines / thyroxine
				T_4‡	T_3	I	Ty_2	Ty_1	C.R.	T_4	T_3	I	Ty_2	Ty_1	C.R.	
Normal thyroid		1	5½	3	—	8	54	27	6	4	—	75	73	62	33	17
		2	17	7	1	8	56	22	6	25	16	99	176	146	48	7
		3	48	11	1	4	45	33	6	56	12	129	412	398	108	7
		4	48	11	2	7	38	35	6	37	32	176	265	305	87	8
		5	121	19	1	5	52	19	5	93	20	110	408	179	70	3
Exophthalmic goiter	Before treatment	6	5½	9	1	8	48	28	5	52	48	274	289	268	88	6
		7	7¼	10	2	10	42	34	4	94	62	543	576	796	117	7
		8	41	21	3	7	40	23	6	184	151	247	268	339	72	2
		9	93	30	5	9	27	21	7	445	194	288	378	450	158	1
	After treatment	6	—	15	2	6	43	29	5	64	50	31	208	221	49	3
		7	—	19	4	5	37	29	6	109	143	18	260	263	77	2
		8	—	21	3	5	39	23	5	83	81	62	149	174	23	2
		9	—	25	5	8	31	21	7	232	81	7	245	216	89	1

* Each figure is an average of two duplicate values.

** Counts/sec./mg. I^{127} corrected to 500-μc. dose and for decay from time of I^{131} administration.

† Average of specific activity (S.A.) of both diiodotyrosine and monoiodotyrosine divided by specific activity of thyroxine.

‡ Abbreviations: same as in Table 1.

COMMENT

The present data indicate that no hitherto unknown iodinated compounds are present in exophthalmic goiters as compared with normal thyroid glands. The qualification must always be made, however, that a substance or substances with the same chromatographic behavior as those of the known compounds may be present but be incapable of resolution by the methods utilized. Our values for I^{127} have not supported the older data that the total iodine concentration of untreated exophthalmic goiters is tremendously reduced. Radioiodine transfer among the iodinated amino acids appears to occur faster in goitrous glands than in normal ones, as was expected. Assuming the tyrosines to be precursors of thyroxine, the ratio of the specific activities of the tyrosines to that of thyroxine should approach unity at some finite time. The ratio was unity in the exophthalmic goiter studied ninety-three hours after radioiodine was administered, but had not approached unity in one hundred and twenty-one hours in the normal gland, again indicating a more rapid rate of thyroxine formation in the thyrotoxic patients than in the euthyroid patients.

The mechanism of the ameliorating clinical effect of Lugol's solution on exophthalmic goiter remains enigmatic. Gutman and co-workers suggested that the thyroid after lugolization returned to normal with respect to the total concentration and distribution of iodine. The involuting action of iodide on exophthalmic goiter is well known. Comparison of thyroid tissue

in the same patients before and after ten days of administration of Lugol's solution disclosed no significant change in histologic appearance, total iodine concentration, and distribution of I^{131} among the four iodinated amino acids. However, there was an absolute and relative increase in iodide content. Three of the 4 patients, nevertheless, showed the customary clinical remission due to Lugol's solution, from which one might infer that the clinical effect of iodide in thyrotoxicosis precedes the involuting action. Thus the histologic involution cannot be the cause of the depression in the abnormally high rate of thyroxine secretion, but rather may result from some other action of iodide on the chemical processes of the gland.

An indication, apart from the clinical evidence, that a slowing of thyroxine formation had occurred in ten days of lugolization is obtained from the specific activity ratios of the tyrosines to thyroxine shown in Table 3. As stated earlier, the specific activity ratio is unity in four days in the untreated goiter. In the tissue from the untreated goiters obtained prior to four days ($5\frac{1}{2}$, $7\frac{1}{4}$ and 41 hours after administration of I^{131}) the ratio varied from 2 to 7, and did not approach unity after ten days during which Lugol's solution was administered. Had iodide no effect on the formation of thyroxine, the specific activity of all of these tissues should have been 1 or less when examined ten days later.

These data, especially those relative to specific activity, are too meager to permit any estimate of rates of hormone formation or turnover, except for the grossest of impressions. Many more data are needed on this aspect of thyroid function in exophthalmic goiter. Since we are not optimistic about the further opportunity to obtain thyroid tissue from patients with untreated thyrotoxicosis, it is our hope that others interested in this problem may pursue it.

SUMMARY

Data on the content, distribution and turnover of iodine in the thyroids of 5 euthyroid persons and of 4 patients with exophthalmic goiter, both in the untreated state and after seven to ten days of iodine therapy, have been obtained by means of the use of I^{131} and column chromatography. No heretofore undiscovered iodinated compounds were observed in either the normal thyroids or the exophthalmic goiters. The mean distribution of iodine in normal glands was found to be as follows: thyroxine, 35 per cent; triiodothyronine, 8 per cent; diiodotyrosine, 25 per cent; and monoiodotyrosine, 17 per cent. That in the untreated goitrous glands was not essentially different. The total iodine content of the exophthalmic goiters was only slightly less than that of normal tissue. The rate of transfer of iodine to thyroxine was apparently increased in the toxic glands.

The administration of Lugol's solution for seven to ten days did not

cause involution, or change the total iodine concentration. There was, however, an increase in the inorganic iodide fraction. The suggestion is made that iodine therapy acts in some manner to slow the formation of thyroxine, after which histologic involution occurs.

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FAMILIAL ADDISON'S DISEASE WITH SPASTIC PARAPLEGIA

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IN HIS extensive review of Addison's disease, Guttman (1) doubted the existence of a definite familial tendency, because of the infrequency of reports of occurrence in more than one member of a family. Familial spastic paraplegia has, however, been described frequently, and the reviews of Rhein (2) and of Paskind and Stone (3) cover some 150 families. Despite this, it remains an uncommon neurologic disorder.

The association of familial spastic paraplegia with Addison's disease does not appear to have been described previously, and it is for this reason, and to record a further example of familial Addison's disease, that these cases are reported.

CASE I

Mr. S.S. In 1935, when 32 years of age this man sought medical advice because of malaise and loss of weight. He was admitted to the hospital for investigation, and was recorded as being of slight build with pigmentation of the face. There were also patches of blue-brown pigmentation on the buccal mucosa. The highest recorded blood pressure was 106/70. There was no abnormality of the central nervous system. Radiographs showed the skull, chest, and abdomen to be normal. The plasma concentration of chlorides was 100 mEq./L. (NaCl, 585 mg./100 ml.).

A diagnosis of Addison's disease was made. He was treated by oral salt mixture, and there appeared to have been considerable improvement.

Six months later he was admitted to a hospital at which clinical trials of "Cortin" were being conducted. Slight but definite pigmentation of the skin and buccal mucosa was again recorded, the blood pressure now being 115/74. Biochemical data of significanc at this time were: blood chlorides 64 mEq./L. (NaCl, 376 mg./100 ml.), blood sodium 124 mEq./L. (285 mg./100 ml.). A standard glucose tolerance test showed a flat blood-sugar curve. On gastric analysis, free acid was present in the stomach. After an initial observation period without added salt, the patient was given a ten-day course of "Cortin," resulting in a slight elevation of blood chlorides to 70 mEq./L. (Na Cl, 410 mg./100 ml.) and apparent subjective improvement.

The patient was subsequently maintained on oral salt mixture, desoxycorticosterone acetate (DCA) intramuscularly and Eucortin, together with implants of DCA in 1943

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and 1946. His progress appears to have been satisfactory until March 1947, when he complained of stiffness of both lower limbs. Neurologic examination showed that he had a spastic gait, with a marked increase in tone in both legs. Lower limb reflexes were grossly exaggerated, with bilateral extensor plantar responses. The abdominal reflexes were absent, and there was wasting of the leg muscles. There was no sensory change, intention tremor, or nystagmus. He refused to be admitted at this time, but in August 1949 he was admitted in what appears to have been a crisis of Addison's disease.

Shortly before this admission a gluteal abscess had developed. Refusing to call medical aid, he had been without DCA for ten days. He was extremely ill and disorientated, and the blood pressure was 95/55. His neurologic status was unchanged, except that early optic atrophy was recorded. There was a large abscess in the left buttock. The cerebrospinal fluid was normal, and the Wassermann reaction in both blood and spinal fluid was negative. Significant biochemical findings were: plasma chlorides 95 mEq./L. (NaCl, 555 mg./100 ml.), serum sodium 128 mEq./L. (295 mg./100 ml.), and serum potassium 4.2 mEq./L. (16.5 mg./100 ml.). Radiographs showed the chest and spine to be normal.

Despite immediate therapy with intravenous glucose-saline and Eucortone, the patient died in twenty-four hours. Unfortunately, permission for autopsy was not granted. His death was considered to have been due to Addison's disease, aggravated by the gluteal abscess. His nervous disability was thought to be disseminated sclerosis.

CASE II

Mr. A.S., aged 40, a younger brother of the preceding patient (Case I), was admitted to the hospital in October 1953 with symptoms of darkening gums, muscular weakness, stiffness in the legs, and of some breathlessness on effort, beginning seven months before. There were no alimentary symptoms and no previous illnesses. A deformity of the feet, of which he had previously been unaware, prevented his serving in a combatant unit in the recent war.

Examination showed an afebrile, thin, listless man with a bronzed skin, and patches of blue-brown pigmentation inside the mouth. Axillary hair and eye lashes were absent, and the hair of the head was soft and scanty. The neck, lungs and external genitalia were normal. The pulse was small in volume and regular, and the heart impulse and sounds were normal. Blood pressure readings varied between 100/65 and 118/74. Positive findings in the central nervous system were: a spastic gait with increased tone and diminished power in the legs; exaggerated lower limb reflexes, bilateral extensor plantar responses, and rigid pes cavus; and pathologically brisk upper-limb and jaw reflexes.

Laboratory investigations: Blood Hb 12.8 Gm./100 ml.; color index 1.14; red cells of normal appearance, apart from slight anisocytosis; white cell count 3,500/cu. mm.; differential white cell count normal. The serum concentration of sodium was 135 mEq./L. (311 mg./100 ml.); plasma chlorides 95 mEq./L. (NaCl, 556 mg./100 ml.); and serum potassium 5.9 mEq./L. (23.1 mg./100 ml.).

With free intake of fluid, the quantity of urine excreted in twenty-four hours was 1,310 ml., containing 7.8 grams of sodium chloride (sodium, 3 grams). The cerebrospinal fluid cell count, Lange curve, and sugar and protein content were normal. Cerebrospinal fluid and blood Wassermann reactions were negative. A fractional test-meal demonstrated normal gastric acidity. The standard glucose tolerances test, showed the blood sugar level to be 70 mg./100 ml. fasting, rising to a maximum of 122 mg./100 ml. In the Kepler test, the value for factor A was 49, but it was noted that only 75 ml. of urine was passed in the three hours following the drinking of 1,020 ml of water. Results of the 48-

hour ACTH test were considered positive, in that there was no significant fall in total eosinophil count or increase in the urinary ketosteroids. Cortisone restored a normal diuretic response to a water load.

Radiographs of the chest showed generalised emphysema with an old scar at the right apex; no calcification was visible in the abdomen, and the spine showed generalized osteoporosis as the only significant abnormality.

Treatment of this patient with cortisone caused an increase in well being, and a lessening of the pigmentation.

FAMILY HISTORY

The father of these brothers died at the age of 65 from secondary carcinomatosis of the liver. Their mother died at the age of 77 from pernicious anemia; no neurologic abnormality was recorded at the time of death. Their brother, aged 46, had had diabetes mellitus for thirteen years and was insulin-sensitive, requiring only 25 units of insulin daily; on examination, he was free from any neurologic disability. Their sister aged 52, was reported by her doctor to have rheumatoid arthritis, without evidence of endocrinopathy or neuropathy. The 7 children of the patients and of their siblings were free from any chronic illness.

DISCUSSION

The diagnosis of Addison's disease seemed adequately established on both clinical and biochemical grounds in these brothers. In the absence of any definite evidence of tuberculosis, it appears probable that the underlying pathology in each case was adrenocortical atrophy. It is possible that the occurrence of diabetes mellitus in the third brother represents another example of an endocrinopathy in this generation.

Smith and Higgins (4) described 2 brothers in whom the diagnosis of Addison's disease was firmly established. They refer to a publication by Curschmann (5) reporting 4 affected brothers, in 1 of whom an autopsy was performed which showed atrophy of both adrenals. Briggs *et al.* (6) reported 2 brothers with Addison's disease, in 1 of whom postmortem examination revealed atrophy of the adrenal cortex. Rolleston (7) stated that he knew of 2 sisters with Addison's disease. The case of a boy thought to have Addison's disease, whose mother had died of this malady, was reported by Borghini (8). Wakefield and Smith (9) found atrophy and hemorrhage in the single adrenal gland of their male patient succumbing to Addison's disease; they state that 2 of his brothers, his father, and his paternal uncle were pigmented. A family of 3 brothers with symptoms of Addison's disease and cutaneous pigmentation was described by Fahr and Reiche (10); autopsy in 1 case showed bilateral adrenocortical atrophy. Croom (11) found undoubted Addison's disease in a girl aged 9, and pigmentation in 2 younger sisters. Fleming and Miller (12) reported frank Addison's disease in a mother whose 4 children were pigmented and subject to attacks of diarrhea and vomiting. No patient in the large series of cases reviewed by Guttman seems to have had affected relatives.

In our search through the literature we have found 9 families in which it seems probable that Addison's disease occurred in more than one member. In the 4 families reported by Curschmann (5), Briggs *et al.* (6), Wakefield and Smith (9), and Fahr and Reiche (10) postmortem records in 1 affected member of each family are available, and in every case the essential finding was atrophy of the adrenal cortex.

The neurologic features in both of the cases reported in this paper were remarkably similar in type and progress, and in each patient the disease became manifest at about the same age. The essential lesion in each instance was spastic paraplegia, but this was associated in one patient (Case I) with absent abdominal reflexes and early optic atrophy, and in the other (Case II) with involvement of the arms and pes cavus. Bickerstaff (13) was impressed by the frequency of optic neuritis in a family of spastic paraplegics he described, and observed that the pes cavus commonly present in his cases was corrected by weight bearing. Similar findings were reported by Garland and Astley (14), who considered the presence of pes cavus to be an important sign in the differential diagnosis between familial spastic paraplegia and disseminated sclerosis, when a strong family history of paraplegia was not obtainable. The upper limbs were affected in 7 families recorded by Rhein (2), and in his "group 5" he described families in which the spastic paraplegia was accompanied by wasting of the leg muscles. The tendency for this disease to appear at about the same age in members of a particular family was a notable feature in some reports (Garland and Astley (14); Dick and Stevenson (15)).

It is difficult to dispute that both brothers had disseminated sclerosis, but in neither was there true dissemination in time, and neither had the more classic signs of this disease. We favor the view, therefore, that they are examples of familial spastic paraplegia, despite the absence of confirmatory neurologic findings in other members of the three generations who were investigated. Such small groups, however, have been included in the reviews of the literature by both Rhein (2) and Paskind and Stone (3).

Psychiatric disturbances accompanying Addison's disease are well recognised, and have been reviewed recently (16, 17). On the other hand, the association of an organic neurologic disorder with Addison's disease is uncommon, and is not mentioned by Guttman (1). Cases of Addison's disease showing evidence of organic cerebral disorder were described by Siemerling and Creutzfeld (18), and by Hampel (19). In both instances, the essential postmortem findings were atrophy of the adrenal cortex, and perivascular infiltration and gliosis of the cerebral hemispheres. Von Neusser and Weisel (20) record a patient with Addison's disease and spastic paraplegia. Van Bogaert and Froehlich (21) found signs of involvement of the extrapyramidal tract in a case of Addison's disease. The association

of diabetes mellitus and Friedreich's ataxia, of which an example has been published recently (Ashby and Tweedy (22)), is well recognised. The occurrence of diabetes mellitus in the third brother of our family is noteworthy in this respect.

The association of Addison's disease and spastic paraplegia in the 2 brothers of this report would appear to be fortuitous. It does not seem possible to postulate a single developmental abnormality which could affect both the suprarenal cortex (mesodermal) and the central nervous system (ectodermal).

SUMMARY

Addison's disease associated with spastic paraplegia occurring in 2 brothers is reported. The paraplegia is considered to be due to a familial degenerative lesion.

Acknowledgment

It is a pleasure to record our gratitude to Dr. C. Gray Imrie of the Royal Hospital, Sheffield, for permission to publish Case II, and for his advice and helpful criticism. For their help in making available to us the clinical records of Case I we should like to thank Professor G. M. Wilson of the Royal Infirmary, Sheffield, and Professor Sir L. S. Davidson of the Royal Infirmary, Edinburgh.

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PITUITARY DWARFISM AND TOXOPLASMOSIS

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HYPOPITUITARISM may involve all pituitary secretions (panhypopituitarism), or may be limited to failure of secretion of one or two hormones. With recent improvement in methods of study, interest in the latter group has grown. One syndrome in this group, however, has been under discussion for many years, namely that of pituitary dwarfism. The growth deficiency is believed to be due to lack of secretion of growth hormone. In most cases the stunting of growth is associated with hypogonadism, whereas thyroid and adrenocortical function are normal, indicating normal TSH and ACTH secretion (1–4). Pituitary dwarfism may be due to a destructive lesion of the anterior pituitary, most commonly a craniopharyngioma (1). In a second group of cases there is no evidence of a destructive lesion; the deficiency is perhaps due to an anlage defect of the eosinophils of the anterior pituitary, analogous to the deficiency of the dwarf Silver mouse (5–7).

The patient whose case is reported here, presented himself because of deficient growth associated with lack of sexual development.

CASE HISTORY

J.D., aged 19, was admitted to The Jefferson Medical College Hospital on October 7, 1953. His mother stated that, following an uncomplicated prenatal course, the delivery of this only child had been difficult, that forceps had been required, and that following delivery no one expected the infant to survive because of listlessness, poor feeding and cyanosis. The infant was born at term, and weighed 7 pounds. The mother further stated that she had foul lochia for from six to eight months. Except for rubeola, varicella and mumps the patient experienced an uncomplicated childhood. He entered school at the age of 6, being 90 cm. tall at that time. Shortly after entering school his parents noticed that he was smaller than his schoolmates, and that he continued to grow slowly, always remaining shorter than other children his age. Throughout his school life the patient excelled in the arts and averaged 80-85 in mathematics. He graduated from high school with an average of 85. The patient did not participate in sports, nor did he show any

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interest in the opposite sex. At the age of 13 his mother noted that he became obese, even though he "did not seem to eat more than usual." Tonsillectomy was performed at the age of 9, without complications. Hives developed whenever the patient took orange juice. Because of poor vision, especially of his left eye, glasses were prescribed when he entered school.

The mother, 46 years old, was 157.5 cm. in height and remembered no illnesses other than childhood diseases. She had had a spontaneous abortion when four months pregnant in 1938, four years after delivery of the patient.

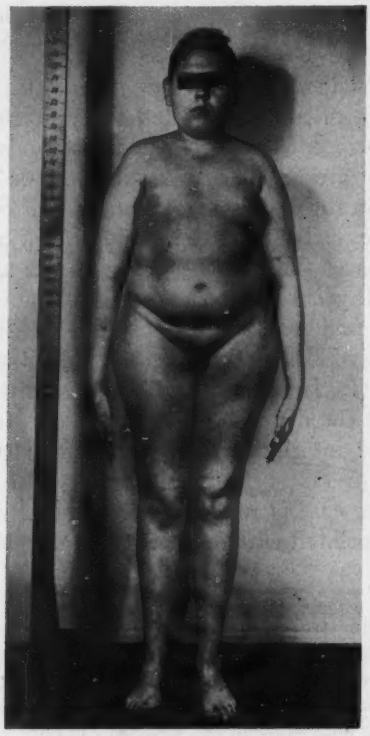

FIG. 1. **J.D.**, age 19. Note immaturity and height.

The father, 45 years old, was 167.5 cm. tall and remembered no significant illnesses The heights of maternal and paternal relatives varied from 147.5 cm. to 180 cm.

On physical examination the patient was found to be 148 cm. tall and to weigh 54.9 Kg. The blood pressure was 110 mm. Hg systolic and 64 mm. diastolic. The temperature during hospitalization fluctuated between 98° and 100° F.; the pulse was 88–94, and respirations 20. He measured 62 cm. from vertex to symphysis and 86 cm. from symphysis to heel. The head circumference was 51 cm., and the measurement from the external occipital protuberance to the glabella was 33.5 cm. The span was 155 cm. The patient, with a chronologic age of 19 years, had the appearance of a child 10 to 12 years old (Fig. 1). He was alert and intelligent, but reticent; his voice was high pitched. The skin was

fine and smooth, and many venules were prominent. There was a tannish fibromatous lesion, 3×2 cm., on the right forearm. The scalp hair was fine. There was neither axillary nor pubic hair, nor was there beard growth. On ophthalmologic examination the discs were ovoid with discrete edges (Fig. 2). There was temporal pallor of the left disc with adjacent disturbance of the retinal architecture. A large area of chorioretinitis obliterated

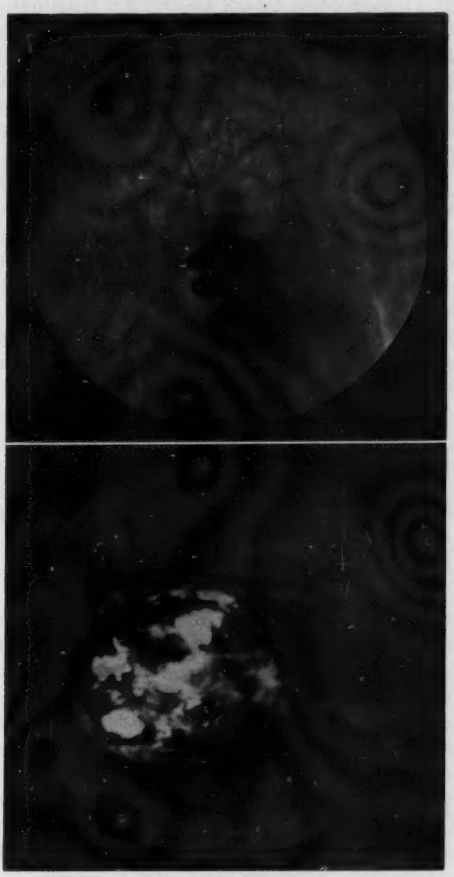

FIG. 2. Funduscopic pictures. Right eye (above); left eye (below). Note areas of chorioretinitis.

the left macular area. There was retinal architectural disturbance of the lateral margins of the right disc, and a similar area of chorioretinitis inferiorly. Vision with and without glasses was O.D. 20/20, O. S. 20/200. The helix of the left ear was bilobed. Examination of the oral cavity disclosed teeth #1, 3, 13, 16, 17 and 32 to be missing, with root stumps in the areas of #6, 19 and 30. There was pseudogynecomastia. Examination of the lungs and heart revealed no abnormalities. The abdomen was obese. The testes were present in the scrotum and measured 1.0–1.5 cm. in diameter. The penis measured 2.2 cm. in

length. On rectal examination the prostate was small. There was a slight degree of thoracic kyphosis. The fingers were tapering.

Laboratory data: The blood count and urinalysis findings were normal. Wassermann and Kahn reactions were negative. On chemical analysis, the concentration of blood urea nitrogen was 10 mg. per 100 ml., creatinine 1.0 mg., calcium 10 mg., phosphorus 3.1 mg. and 3.25 mg., alkaline phosphatase 4.5 and 2.6 Bodansky units, plasma protein 6.57 grams (A/G ratio, 4.27/2.3), fasting blood sugar 80 mg., and cholesterol 240 mg. per 100 ml., serum sodium 132.5 mEq. per liter potassium 4.02 mEq., and chlorides 100 mEq. The following blood sugar values were obtained during an intravenous glucose tolerance test: fasting 100 mg. per 100 ml., half hour 220 mg., one hour 140 mg., two hours 103 mg., three hours 105 mg., four hours 75 mg., five hours 77 mg., and six hours 88 mg. per 100 ml. The insulin tolerance test, using 0.1 unit of regular insulin per kilogram intravenously, resulted in the following blood sugar values: fasting 81 mg. per 100 ml., twenty minutes 47 mg., thirty minutes 50 mg., forty-five minutes 58 mg., sixty minutes 70 mg., ninety minutes 75 mg., and one hundred and twenty minutes 78 mg. per 100 ml. The BMR was −9 per cent, the serum protein-bound iodine level was 7 micrograms per 100 ml.; and the I^{131} tracer study showed a thyroidal uptake of 22 per cent in twenty-four hours. Twenty-five units of ACTH intramuscularly produced a drop in eosinophils from 288 per cubic millimeter to zero. Urinary oxysteroid excretion was 6 mg. per twenty-four hours.[1] Gonadotropin excretion was less than 12 mouse units per twenty-four hours, and 17-ketosteroid excretion was 4.6, 5.1 and 3.4 mg.-equivalents of androsterone per twenty-four hours. A testicular biopsy revealed normal testicular tissue but no spermatogenesis. A roentgenogram showed the chest to be normal. Roentgenograms of representative bones showed over-all demineralization and a bone age of 12–14 years. Roentgenograms of the skull disclosed multiple intracranial calcifications, occurring most frequently in the occipitoparietal area (Fig. 3). The sella turcica was normal. On roentgenographic examination, the mother's skull was interpreted as being within normal limits. Excisional biopsy of the lesion of the right forearm revealed a cutaneous fibroma.

Psychologic examination (Dr. Olive Morgan): Intelligence—Wechsler-Bellevue intelligence scale, Form I; verbal quotient, 101; performance quotient, 65; full scale quotient, 83. The wide discrepancy between the verbal and performance quotients was significant. Among the verbal abilities, all were high average except logical thinking, which showed serious deficiency. Among the performance tests, all were in the subnormal range, with conceptualization lowest. To determine if these very low scores in the performance range indicated organic dysfunction, appropriate tests were used, but the results were negative. Apparently the mother had been very protective. She accompanied the boy to the clinic, took his coat and supervised every movement. Although physically handicapped to some degree, this did not seem to account for his lack of performance development. His verbalization, although knowledge of vocabulary was good, was exceedingly immature.

Tests for toxoplasmosis: The funduscopic picture together with the finding of multiple intracranial calcifications suggested the diagnosis of toxoplasmosis. Consequently the following additional studies were carried out. Dr. Leon Jacobs, National Institutes of Health, kindly performed the cytoplasm-modifying and complement fixation tests for toxoplasmosis with the following results: on October 20, 1953, the results of the *Toxo-*

[1] We are indebted to Dr. J. Schneider, of this department, for performing this test with a method based on enzymic hydrolysis and the use of the Porter-Silber reaction. Normal values range from 5 to 9 mg. per twenty-four hours.

plasma dye test were positive in dilutions of 1:4096, and results of the complement fixation tests were negative. On January 7, February 18 and April 22, 1954, the titers of the dye test were 1:64, and there was no complement-fixing antibody. Examination of the mother's serum revealed a dye test titer of 1:64, and negative results with a complement fixation test.

A lumbar puncture was performed, and clear fluid was obtained; there were no cells. The concentration of spinal fluid protein was 16 mg. per 100 ml. Five mice were immediately inoculated intraperitoneally with 0.1 ml. of the spinal fluid. Serial inoculations were carried out on three occasions, using the same number of mice. No lesions resulted. No *Toxoplasma* organisms were observed in wet or in stained films.

FIG. 3. Roentgenogram of skull. Note multiple areas of calcification.

Following study in the hospital the patient was discharged, and received a course of chorionic gonadotropin (1,000 units intramuscularly three times weekly for a period of six weeks). He was observed for seven months. He noted deepening of his voice, development of acne, pubic and axillary hair, a growth spurt, and enlargement of his penis. The urinary gonadotropin excretion rose to 55 and 17 mouse uterine-weight units per twenty-

four hours (two determinations), and the 17-ketosteroid excretion to 6.2 and 6.5 mg.-equivalents of androsterone per twenty-four hours (two determinations).

DISCUSSION

The findings in this patient appear to fulfil criteria for the diagnosis of pituitary dwarfism (1, 2, 3). The patient's growth was apparently retarded and there was failure of pubertal maturation. That the latter was due to pituitary deficiency is indicated by the lack of gonadotropin excretion, the immature histologic picture of the testis, and the response to treatment with chorionic gonadotropin. The deficiency of growth hormone secretion could not be demonstrated by hormone assay, but could be deduced indirectly. Isolated failure of pituitary gonadotropin secretion (pituitary hypogonadotropism) causes eunuchoidism, with excessive growth in length rather than stunting of growth, and with disproportionate length of the extremities (eunuchoid proportions). The combination in the present case, of hypogonadotropism with retarded growth suggests a second hormonal deficiency. The fact that this patient's thyroid function was normal (manifested by the normal basal metabolic rate, normal serum protein-bound iodine level, and normal thyroidal I^{131} uptake) leaves, by exclusion, only a growth hormone deficiency as a probable explanation.

As recorded in the case history, this patient's response to insulin was normal. It is well known that the hypophysectomized animal as well as the patient suffering from panhypopituitarism shows increased insulin sensitivity (6, 8); under such circumstances it is difficult to tell to what extent growth hormone deficiency or ACTH deficiency is responsible for the insulin sensitivity. Both growth hormone and adrenal C_{11}-oxygenated steroids are known to be insulin antagonists. However, there are not enough unequivocal observations on record to permit a statement as to whether growth hormone deficiency, in the presence of normal ACTH-adrenal cortex activity, regularly causes marked insulin sensitivity. We therefore do not believe that the normal insulin response in the present case would militate against the diagnosis of pituitary dwarfism, *viz.*, deficiency of both pituitary growth hormone and gonadotropin.

The diagnosis of congenital toxoplasmosis in this patient is based upon the findings of cerebral calcifications, chorioretinitis, and the positive results of laboratory tests (9–20). Values obtained with the cytoplasm-modifying or dye test are considered significant when the titer is 1:16 or above; such titers are usually detectable within two weeks after the onset of illness, and may rise to 1:10,000 (16, 21). Significant titers may persist for years or even for life. Complement-fixing antibodies appear later in the course of illness, at about thirty days, and disappear usually within two to four years (10, 16). Persistence of antibodies in high titer does not

indicate active infection; active or acute infection is characterized by absence of complement-fixing antibody, associated with rising titers of the cytoplasm-modifying antibody (16). However, significant titers in the dye test, associated with negative results in the complement fixation test, are observed not only in acute or active disease but also in cases of infection acquired in the past (16). In infants, absence of complement-fixing antibody and high titers with the dye test, in the presence of high titers of both in the mother, signify active infection (16). With survival, the infant may later show rising complement fixation titers. Positive skin reactions may develop following injection of antigen (22–24). The skin reaction, though specific, is not always positive in active or past infections, and cannot differentiate persons with low, high or rising titers of cytoplasm-modifying antibodies. Skin sensitivity was not tested in this patient. Short of recovering the parasite, the findings of chorioretinitis, multiple cerebral calcifications, and positive results with the dye test in both patient and mother indicate that in all probability toxoplasmosis developed in utero as the result of an asymptomatic infection of the mother (14–16; 25–27). Cerebral calcifications and chorioretinitis are not believed to occur in the acquired type, but only in the congenital type (16). The negative findings with complement fixation tests, and the positive cytoplasm-modifying titers in this particular patient fit well with the data reported in the literature (16). The results of the tests, as observed over a period of seven months, suggested that the disease was neither acute nor recent. The positive test results in both mother and son, plus the physical findings, satisfied the requirements for a diagnosis of congenital toxoplasmosis.

Although the disease appeared inactive, it was decided to inoculate some mice with cerebrospinal fluid. The anticipated negative result did not militate against the diagnosis of toxoplasmosis. It supported the assumption that the disease, at the time of observation, was inactive.

The most commonly recognized clinical manifestations of toxoplasmosis result from congenital infections, acquired during an unrecognized infection of the mother. Intra-uterine death followed by abortion, prematurity or term delivery may occur after infection, immediately or later in life (13–16; 27). Patients who survive congenital infection may present microcephaly, hydrocephaly, psychomotor retardation, symptomatic epilepsy, chorioretinitis and cerebral calcifications. Infections acquired later in life are not similarly apparent, but some patients present the clinical picture of rickettsial or leptospiral disease, and even death may ensue (11, 13, 15, 16, 17, 25, 26, 28).

At autopsy, involvement of most organs of the body has been found (10, 13, 16, 18–20). Cerebral infection appears to be the cause of the microcephaly, hydrocephaly, psychomotor retardation and symptomatic

epilepsy observed in some cases. In view of the widespread lesions produced by infection, pituitary involvement is possible. In our case the cerebral calcifications were miliary and widespread; none was seen roentgenographically within the sella turcica. The absence of demonstrable calcium deposits, however, does not rule out an infection of the pituitary. The possibility should also be considered, that involvement of hypothalamic centers might have caused pituitary hypofunction.

The simultaneous occurrence of toxoplasmosis and pituitary dwarfism in one patient may be fortuitous, but in the absence of any other obvious cause for the latter, it appears probable that infection with *Toxoplasma gondii* was the cause of the endocrinopathy.

CONCLUSION

A case of pituitary dwarfism (inhibition of growth and sexual retardation) in a 19-year-old male showing evidence of congenital toxoplasmosis, is presented. The possibility is discussed that *Toxoplasma* infection of the pituitary or hypothalamus may have been the cause of the pituitary hypofunction.

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Editorial

BLOOD ACTH*

THE development of methods for the determination of hormone titers in the peripheral blood is a goal of considerable theoretical and practical importance to the endocrinologist. The goal is exceedingly difficult to attain, for minute quantities of most hormones induce a response in a target gland or in an effector cell. Furthermore, analysis is carried out on a sample of blood which contains but a small fraction of the total quantity of hormone in the circulation or in total body water at any instant. The effectiveness of low concentrations of hormone constantly maintained is illustrated by an experiment in man in which 20 units of ACTH injected intravenously over thirty seconds produced no detectable increase in adrenocortical activity, whereas the same dose given by infusion over forty-eight hours produced a near-maximum response (1). Obviously, assays of hormones in peripheral blood require extremely sensitive chemical or biologic methods. Added to these difficulties is the fact that blood is a complex mixture which demands a high degree of specificity in the assay method or a high degree of purification in a process designed to isolate the hormone. The task is formidable but progress is being made, as exemplified by the development of methods for the determination of corticosteroids (2, 3, 4) and of ACTH (5, 6) in the peripheral blood of man. Blood ACTH has been demonstrated to be increased in Addison's disease, in bilaterally adrenalectomized subjects and in subjects with the adrenogenital syndrome (5–8). Treatment with cortisone results in a reduction of these high titers to nondetectable levels (7, 8). These observations fit accepted concepts and appear to be generally accepted among investigators in the field. However, marked disagreement exists as to the concentration of ACTH in the blood of normal subjects and it is to this aspect of blood ACTH that the editorial is directed.

The interested reader will find descriptions and critical analyses of methodology in two communications in this journal (5, 6). Adrenal ascorbic-acid depletion in the hypophysectomized[1] rat has been the method

* Work from the author's laboratory cited in this review was supported by grants from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

[1] Bioassays conducted in animals with intact hypophyses are worthless.

commonly employed for assay of blood ACTH.[2] The application of this method to the determination of blood ACTH requires strict adherence to the requirements for accurate reliable assays, since in many instances one has to operate at the lower range of sensitivity of the method.[3]

A more direct approach to the determination of the quantity of ACTH in blood is the measure of the capacity of ACTH to increase the rate of secretion of adrenal steroids in the hypophysectomized animal. A preliminary report has appeared, which indicates that this can be accomplished in the dog (12). Additional observations based on this method are anticipated with interest.

Preparation of blood for intravenous injection into the assay animal has been carried out in several ways. The plasma may be separated and injected without processing into the test animal.[4] An acid-acetone procedure has been employed to concentrate hormone activity. In the author's experience the acid-acetone technic almost invariably fails to give acceptable recovery of ACTH from plasma. The method described by Astwood and co-workers for the concentration of ACTH (13), as modified by Bartholomew (14), has been adapted to the assay of blood ACTH (15). On the basis of the author's experience with various methods for determination of blood ACTH concentration, the conclusion is reached that the oxycellulose procedure is distinctly superior.

According to Paris and co-workers (5), administration of plasma from normal human subjects induces no significant depletion of adrenal ascorbic acid. Eluates of oxycellulose equivalent to 40 ml. of blood induce no significant response in the test animals (6). The results have been interpreted to mean that the concentration of ACTH under optimal conditions is less than 0.5 milliunit (mu)[5] per 100 ml. of blood.[6] These negative

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[2] Reports have appeared in the literature to the effect that the adenohypophysis elaborates two or more types of ACTH—one that induces adrenal ascorbic-acid depletion, one that induces an increase in adrenal weight, and another that induces an eosinopenia. A detailed analysis of this aspect of adenohypophysial physiology cannot be made in this review. Suffice it to state that preparations which are potent in inducing adrenal ascorbic-acid depletion produce in man metabolic changes characteristic of hypercorticism (9), increase the rate of excretion of urinary corticoids (10), and increase the concentration of corticoids in the blood (11).

[3] In gravimetric terms the concentration of ACTH in the blood in Addison's disease, a disorder in which the level of blood ACTH is elevated, is of the order of 1×10^{-8} grams per 100 ml. blood!

[4] If the plasma cannot be injected immediately, it must be stored in the frozen state, or lyophilized.

[5] A milliunit equals one-thousandth of a U.S.P. unit of ACTH.

[6] The adrenal ascorbic-acid method varies in sensitivity among laboratories, and from one day to the next in the same laboratory. In the author's experience 0.25 mu of standard ACTH almost invariably induces a significant response. Hence, if a 40-ml. equiva-

observations are in striking contrast to those reported by a number of investigators (16–21) who claim that ACTH is present in a concentration of 30 to 200 mu per 100 ml.! Although one can summarily dismiss reported data with a statement to the effect that faulty bioassay[7] technic,[8] is the reason for the high concentrations reported, more tangible reasons are desirable. Fortunately, evidence is now available from related phases of the subject at hand to support the conclusion that the concentration of ACTH is less than 0.5 mu per 100 ml. of blood under optimal conditions.

1. Injection of 100 ml. of blood from a normal nonstressed dog does not induce an increase in the steroid output of the adrenal of a hypophysectomized recipient dog[9] (12). A response is induced by a single dose of 2.5 mu of ACTH.

2. An estimated concentration of less than 12 mu of ACTH per 100 ml. of plasma induces a maximum response and 3 mu per 100 ml. induces a submaximal but definite response of the human adrenal cortex, as measured by the metabolic changes associated with infusion of the hormone at various dose levels[10] (1).

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lent of blood induces no response, it may be stated that the concentration of ACTH is less than 0.5 mu per 100 ml.

[7] Sources of error in bioassay include: 1) Incomplete hypophysectomy of test rats. 2) Contamination of glassware with potent preparations of ACTH (1 microgram of purified ACTH has an activity equivalent to 100 mu). A room should be set aside for analysis of blood. Potent preparations of ACTH should not be assayed in this room. A useful check on contamination is the assay of 0.9 per cent sodium chloride solution with each set of analyses. 3) Failure to check sensitivity. The sensitivity of the bioassay varies from laboratory to laboratory and from day to day in the same laboratory. In the author's laboratory at least one dose (0.25 mu), and in most instances three doses (0.25, 0.5 and 1.0 mu) of standard are run with the unknowns.

[8] Acid-acetone treatment or oxycellulose processing (21) might conceivably "activate" an inactive form of ACTH in blood. The following evidences are against such a possibility. Acid-acetone fractions have invariably failed to induce ascorbic-acid depletion in the laboratory of Albert *et al.* (5) and in the laboratory of Sayers *et al.* (6). The oxycellulose method (6) has given results in man which confirm those obtained with unfractionated plasma (5). In the adrenalectomized rat, estimates of blood ACTH determined by the oxycellulose method are in excellent agreement with those obtained by direct transfusion of whole blood (15).

[9] The oxycellulose technic indicates that the concentration of ACTH in the blood of nonstressed dogs and nonstressed rats is less than 0.5 mu per 100 ml.

[10] Intravenous infusion of ACTH over eight hours in a dose of 20 units produced a maximum increase in urinary 17-ketosteroids, a maximum depletion of circulating eosinophils and maximum metabolic changes. If the half-life of ACTH is fifteen minutes (we have reason to believe it is shorter than this figure), the total amount of ACTH in the body at any instant which would be attained with an infusion rate of 40 mu per minute (20 units in eight hours) is 600 mu (half-life times the rate of infusion). If the hormone is confined to the vascular compartment, then the concentration of ACTH would be 12 mu per 100 ml. This is a high estimate.

3. An estimated concentration of less than 20 mu of ACTH per 100 ml. of plasma induces a maximum response and 1 mu per 100 ml. induces a submaximal but definite response of the human adrenal cortex, as measured by alterations in the level of corticoids in the peripheral blood (11). (See footnote 10 for calculation of estimate.)

In summary, the exceedingly high titers of ACTH reported to be present in the blood of normal subjects under optimal conditions are difficult to reconcile with established facts. Concentrations of 30 to 200 mu of ACTH per 100 ml., maintained for a few days, would be associated with a marked degree of hypercorticism.

We shall now turn our attention from optimal environmental conditions to stressful situations. Bornstein and Trewhella (16) have reported extraordinarily high concentrations (337 ± 35 mu per 100 ml.) in the blood of subjects following surgical trauma. However, since these same workers have reported high concentrations under optimal conditions, the author of this review is unwilling to accept these values as representative of blood ACTH concentration after application of noxious stimuli.

Detectable quantities of ACTH have been reported to be present in the plasma of a patient with severe dysmenorrhea and in the plasma of 2 patients with pheochromocytoma during the course of paroxysmal hypertension (5).

The administration of the equivalent of 20 to 40 ml. of blood obtained from 2 patients with miliary tuberculosis and processed by the oxycellulose method did not induce a significant degree of depletion of adrenal ascorbic acid. In only 1 of 3 patients who were accidentally scalded, was there a detectable quantity of ACTH in the blood. No ACTH was detected in the blood of healthy male subjects exposed to low atmospheric pressure (18,000 feet for two, fifteen or forty-five minutes), to heat (120° F. for fifteen, or for forty-five minutes), or to the combination of these two environmental disturbances (22).

The variable results which have been obtained in man subjected to noxious stimuli may reflect certain characteristics of adenohypophysial activity. Two minutes after scald, the intact rat invariably exhibits a relatively high titer of ACTH. Six minutes after scald, the concentration of ACTH has returned to the pre-scald nondetectable level. These experiments in the rat emphasize the importance of the time relationship when studying adenohypophysial ACTH activity in response to a noxious stimulus.

Application of stress may induce an increase in the blood ACTH level which effectively stimulates the adrenal cortex, but which is not detectable by the assay method. For example, hemorrhage in the intact dog is associated in a number of instances with barely detectable quantities of blood

ACTH. It is possible that all dogs subjected to bleeding exhibit an increased rate of discharge of ACTH from the adenohypophysis, but the blood level of ACTH is below the limit, or just at the limit of sensitivity of the method.

Obviously, assay of ACTH in the blood of man has definite limitations. It is exacting and time-consuming, especially if one is willing to meet the criteria which have been established for reliable assay technic. A relatively large volume of blood is usually required, which introduces a barrier to the application of the technic to a person subjected to severe trauma and to experiments designed to study time relationship, in which there is need for repeated sampling.

The method is not sensitive enough to detect ACTH in the blood of normal subjects under optimal conditions, nor is it sensitive enough to detect consistently an elevation in ACTH titer subsequent to the application of a noxious stimulus. We must continue to seek ways to increase the sensitivity of the method, for there is obviously a great need for information on the activity of the human adenohypophysis at a given instant following application of various intensities of stimuli.

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Letters to the Editor

THE ISOLATION OF 11-DEHYDROCORTICOSTERONE, CORTICOSTERONE AND TETRAHYDRO COMPOUND S (PREGNANE-3α,17α,21-TRIOL-20-ONE) FROM THE URINE OF A MAN RECEIVING CORTICOTROPIN*

TO THE EDITOR:

11-Dehydrocorticosterone (compound A), corticosterone (compound B) and tetrahydro compound S (pregnane-3α,17α,21-triol-20-one) have been isolated from the pooled urine of a man with rheumatic fever receiving 10 units of intravenous corticotropin daily over a period of five days. A semi-quantitative estimate indicated that a 24-hour urine collection contained 56 μg., 45 μg. and 450 μg., respectively, of these substances.

The technics employed and their limitations have been described previously (1). Initial separation was obtained by paper chromatography in the toluene-propylene glycol system. The substances suspected of being compounds A and B were separated by re-chromatography in methylcyclohexane-propylene glycol, which readily separated them and other alpha-ketolic substances which move together in the toluene-propylene glycol solvent system. Both compounds absorbed ultraviolet light at 245 mμ and reacted with the blue tetrazolium reagent, indicating an alpha-beta unsaturated ketone structure and an alpha-ketol, respectively. After acetylation, both of these compounds showed rates of movement comparable to those shown by authentic materials during chromatography in the methylcyclohexane-propylene glycol system. The results were negative with both compounds in the phenylhydrazine reaction of Porter and Silber (2). The acetates were further purified by chromatography on a silica gel-Celite column. The spectrum in sulfuric acid (3) of the isolated compound A showed maxima at 288, 350 and 410 mμ, in agreement with that of an authentic sample. The isolated compound B showed a quantitative fluorescence value equivalent to that calculated from the ultraviolet absorption at 245 mμ. The maxima of the spectrum in sulfuric acid were

* This investigation was supported in part by a research grant H-922 from the National Heart Institute, of the National Institutes of Health, Public Health Service and by a grant from the American Heart Association.

found at 284, 330, 375 and 440 mμ, in agreement with the known spectrum.

Tetrahydro S, which separated in the same zone as cortisone in the toluene-propylene glycol system, was purified by re-chromatography in the benzene-formamide system and in the methylcyclohexane-propylene glycol system after acetylation. The rate of movement in both instances agreed with that of the known compound. The material did not absorb ultraviolet light at 245 mμ but did show a reaction with blue tetrazolium. A positive result was obtained in the phenylhydrazine reaction of Porter and Silber. The spectrum in concentrated sulfuric acid showed peaks at 310 and 410 mμ, in agreement with the known spectrum.

On infrared spectrometry (performed through the courtesy of Dr. Thomas F. Gallagher of the Sloan-Kettering Institute for Cancer Research, Memorial Center, New York) the findings substantiated the identification of each of the compounds.

The isolation from urine of 11-dehydrocorticosterone has not been reported previously. The isolation of corticosterone is a confirmation of the previously reported (4) isolation of this material in this laboratory which, however, due to lack of material had not been substantiated by infrared analysis. Tetrahydro S has previously been isolated in this laboratory from the urine of a woman with virilization due to adrenal carcinoma (5), and by Rosselet *et al.* (6) from the urine of 2 patients with Cushing's syndrome due to adrenal carcinoma. Tetrahydro S has been described as a major alpha-ketolic metabolite of compound S (1, 6).

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MELANOCYTE-STIMULATING HORMONE EXCRETION DURING PREGNANCY

TO THE EDITOR:

In view of the opinions on the excretion of melanocyte-stimulating hormone (MSH) during pregnancy expressed by Lerner, Shizume and Bunding, *J. Clin. Endocrinol. & Metab.* **14:** 1463–1490 (Dec.) 1954, we wish to draw attention to our investigations (1, 2) published in 1952 and

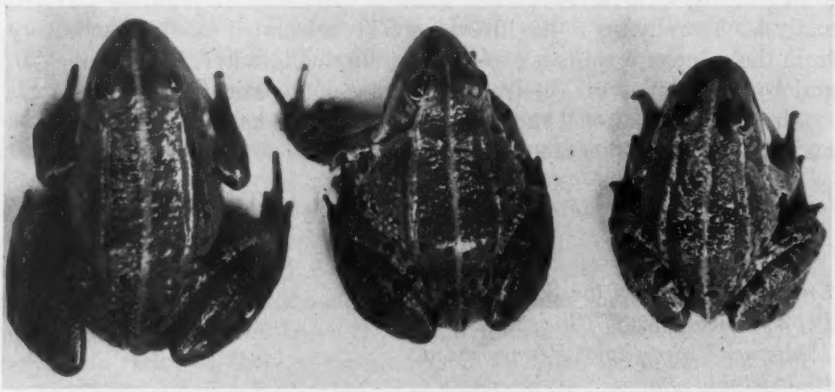

FIG. 1. The two frogs on the left are injected with 2 cc. of pregnancy urine. Note the darkening of the hindpart of the body (pregnancy discoloration; Konsuloff's pregnancy test). The third frog is an uninjected control.

1953 in which it was shown that the hypophysectomized *Rana esculenta* has available two ways of discoloration:

1. *Total discoloration,* in which the entire body of the frog becomes darker.

2. *Pregnancy discoloration,* or *the Konsuloff pregnancy reaction,* in which only the hind part of the body of the frog becomes darker. The reason underlying the name of this reaction is twofold. It is based on the fact that this type of discoloration results from the injection of pregnancy

urine, of pregnancy serum, of aqueous extract of young placentae, of very pure human chorionic gonadotropin, or of pregnant mare's serum. This color pattern (Fig. 1) is seen in the pregnancy test devised by Konsuloff in 1934 (3).

Total discoloration is obtained following injection of melanocyte-stimulating hormone (MSH) (intermedin). Even after very small doses of MSH it is not possible to elicit the pregnancy discoloration. Furthermore, the possibility that there exists an overlapping between MSH and the pregnancy factor is refuted by the fact that the activity of all the substances producing the pregnancy pattern is destroyed by boiling for ten minutes at pH 7, whereas MSH retains its chromatophorotropic capacity after having been submitted to the same treatment.

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A WATER-SOLUBLE PREPARATION OF HYDROCORTISONE FOR CLINICAL USE

To the Editor:

In the course of investigating the urinary excretion and renal clearance of the metabolites of various corticosteroids, the properties of a water-soluble ester of hydrocortisone (the sodium salt of hydrocortisone hemisuccinate[1]) were studied. The data obtained will be reported in detail later. The present note is intended to point out that this water-soluble compound appears to meet a clinical need.

Hydrocortisone hemisuccinate was administered intravenously over a thirty-minute period to a human subject without adrenals. A plot of the

[1] We are indebted to the Upjohn Company Kalamazoo, Michigan, for providing this preparation as U-4905 for clinical trial. It will be marketed under the name of Solu-Cortef.

urinary excretion rate of "total" urinary 17-hydroxycorticoids (butanol-extractable Porter-Silber chromogens) revealed an exponential decay curve characterized by a half-life of four to five hours. This half-life agrees very closely with Hellman's data on the excretion of hydrocortisone 4-C^{14}(1). Within the limits of our method, excretion of the hemisuccinate compound was identical with that of hydrocortisone similarly administered. When given intramuscularly the pattern of excretion was again the same, indicating that adsorption of the hemisuccinate from the extravascular depot was complete within thirty minutes. In addition, the proportion of free (chloroform-extractable) to "total" 17-hydroxycorticoid metabolites was the same, within the limits of experimental error, for the hemisuccinate and for free hydrocortisone given by either the intravenous or intramuscular route. These results imply that the two compounds are metabolized by the liver and excreted by the kidney in a similar manner.

In man the eosinopenic activity of this compound proved to be of the same order of magnitude as that of equimolar quantities of hydrocortisone. This biologic equivalence agrees with results of others using the Ingle work test in rats (2). Intramuscularly administered it has been used successfully, in doses comparable to the hydrocortisone requirement, for treatment of disseminated lupus erythematosus in relapse, acute bronchial asthma, the crisis of Addison's disease, and for replacement therapy during and after bilateral adrenalectomy. No local reactions occurred at injection sites nor were the injections unduly painful.

This preparation meets a real need for a form of hydrocortisone which produces high blood concentrations quickly and is suitable for intramuscular use. If fairly constant high blood levels of hydrocortisone are desired the preparation must be administered every six to eight hours, since it is rapidly absorbed from intramuscular depots and excreted in the urine.

Since the hemisuccinate equivalent to 100 mg. of hydrocortisone can be given by direct intravenous injection in a small amount of fluid (2 cc.) without the use of infusion apparatus, the new preparation is most practical and useful in extreme emergencies and in shock-like states when absorption from extravascular sites is uncertain.

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A METHOD FOR THE ESTIMATION OF URINARY FORMALDEHYDOGENIC LIPIDS, WHICH IS FREE FROM FORMALDEHYDE RETENTION*

To the Editor:

Hollander *et al.* (1), Paterson and Marrian (2, 3) and Wilson (4) have called attention to the fact that in certain urinary extracts incomplete recoveries of formaldehyde are realized from added steroids subjected to periodate oxidation under the usual conditions. The extent of such "trapping" of the liberated formaldehyde may be as much as 70 per cent. No entirely satisfactory explanation of this phenomenon has yet been advanced.

In view of the apparent importance of corticosteroid metabolites (5) which are formaldehydogenic, but are neither reducing substances nor Porter-Silber chromogens, it is probable that the estimation of urinary formaldehydogenic lipids will continue to have great value in the study of adrenocortical function.

It is the purpose of this communication to call attention to a procedure which has been used in this laboratory for nearly three years, and which does not appear to be subject to the drawback of "trapping."

The method is essentially that recently described (6) in which triethyl phosphate is employed as solvent for the reaction mixture and the oxidation and diffusion steps are combined. It has been found that the Conway units may be sealed most conveniently as follows: All of the reagents except the periodic acid are added to the dishes. The top edge of the dish is then warmed for about one-half minute with a small inverted hot plate. The periodate is added and the unit is sealed by pressing a sheet of Parafilm (Grade M) between the dish and its cover. This expedient eliminates the tedious degreasing of the units which is required when petrolatum or other greases are used.

* Publication No. 847 of the Cancer Commission of Harvard University.

This work was supported by grants from the National Cancer Institute, United States Public Health Service (Grants #1393-C, C-2, C-1048), a grant from the American Cancer Society recommended by the Committee on Growth of the National Research Council (H 158), and a grant from the Jane Coffin Childs Memorial Fund for Medical Research.

The data which are shown in Figure 1 consist of 40 assays of added desoxycorticosterone (20–30 μg.) and tetrahydrocortisone (30 μg.) in urinary extracts obtained from 8 patients with various diseases and under various therapeutic regimens. The mean formaldehyde recovery from added

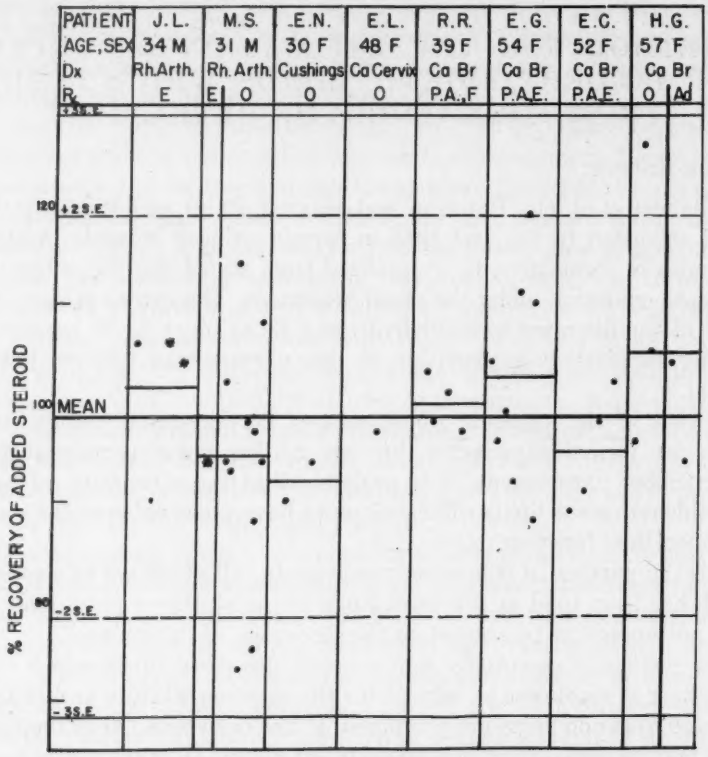

FIG. 1. *Statistical analysis of recovery experiments.* Explanation of symbols: Dx, diagnosis; Rx, therapy; Rh. Arth., rheumatoid arthritis; E., (compound E) cortisone; Ca Cervix, cancer of cervix; Ca Br., cancer of breast; P. A., post-adrenalectomy; F., (compound F) hydrocortisone; Ad, 4-androstene-3, 17-dione; S. E., standard error of single determination; * tetrahydrocortisone used instead of desoxycorticosterone; the horizontal lines are the mean values for individual patients.

corticosteroid was 99 per cent, and the standard error of a single determination was 10 per cent. Analysis of variance revealed that the between-sample variance was not significantly different from the within-sample variance ($p>0.2$). It is evident from Figure 1 that the recoveries were

normally distributed about the mean value and that formaldehyde retention did not occur in this series of experiments.

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A COMPARISON OF URINARY ZIMMERMANN CHROMOGENS BY GIRARD FRACTIONATION AND BY ALLEN'S METHOD OF CORRECTION

TO THE EDITOR:

Precise determinations of urinary 17-ketosteroids have in the past involved separation of ketones from nonketones by means of the Girard reaction. It occurred to us that Allen's method of correction (1) might provide a more direct avenue of approach. The following experiment shows this to be true.

A 100-ml. specimen of urine, to which had been added 10 ml. of 12 N sulfuric acid, was refluxed gently for fifteen minutes. The cooled hy-

drolysate was extracted four times with 0.2 volume of ethylene chloride. The extract was washed with sodium hydroxide and water and evaporated to dryness. Ketonic fractions were obtained by the method of Pincus and Pearlman (2). Assays were performed by the method of Klendshoj *et al.* (3). Optical densities at 440, 520 and 600 mμ were measured. The concentrations of the crude and ketonic fractions, as dehydroisoandrosterone, were calculated by the conventional method, using the optical density at 520 mμ. The corrected values for the crude fraction were calculated by Allen's method, using the formula:

$$OD_{corr} = OD_{520} - 1/2(OD_{440} + OD_{600}).$$

Absorption spectra were obtained on a Beckman spectrophotometer, model DK.

Table 1 shows a comparison of the values for the crude and ketonic

TABLE 1. COMPARISON OF 17-KETOSTEROID LEVELS IN THE CRUDE AND KETONIC FRACTIONS WITH THE LEVEL IN THE CORRECTED CRUDE FRACTION

Urine sample No.	17-Ketosteroids (mg./L.)			
	a, Crude	b, Ketonic	c, Corrected	d, (c/b)
1	12.46	5.48	8.35	1.52
2	15.10	7.35	7.27	0.99
3	12.90	9.46	10.40	1.10
4*	4.30	3.18	3.60	1.13
5	13.80	9.45	11.60	1.23
6*	2.96	1.30	1.99	1.53
7	6.53	2.66	3.91	1.47
8*	10.90	7.05	8.30	1.18

* Washed by NaOH pellets.

fractions with that for the corrected crude fraction. It is clear that there is good correlation between the values obtained by calculation and those obtained by the more laborious chemical fractionation. That the corrected values are slightly higher than those obtained by the Girard reaction may be due in part to incomplete recovery, since experimental evidence indicates a 5 to 10 per cent loss of ketone.

More difficult to assess from a quantitative viewpoint is the effect of ketonic impurities in the Girard fractions. As may be seen from curve K of Figure 1, the urinary ketonic fraction contains substances which absorb more strongly at low wave lengths than does dehydroisoandrosterone. This is not surprising, since Wilson (4) has reported that 3-monoketones,

diketones and triketones of the pregnane and allopregnane series, as well as corticoids, have either the same or higher absorption at 420 mμ than at 520 mμ. Thus it is apparent that the common practice of accepting the values obtained for ketonic fractions as the absolute value of urinary 17-ketosteroid excretion is unwarranted. As in the case of the Allen cor-

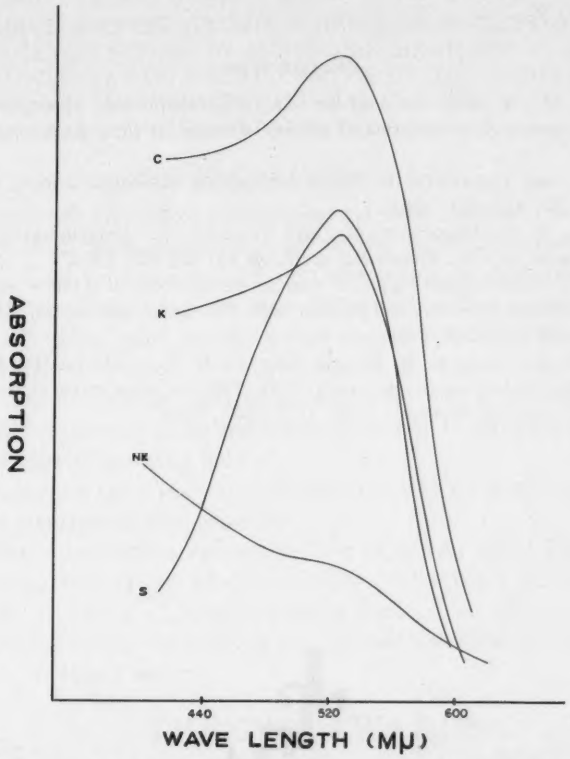

FIG. 1. Absorption spectra of crude (C), ketonic (K) and nonketonic (NK) fractions of neutral urine extract and of 30 micrograms of dehydroisoandrosterone (S).

rection, this method merely permits a closer approximation to the true excretion. Although not shown in Figure 1, the curves for the fractions from the other seven urines were essentially similar, as were those from six of the urines in which the method of Callow *et al.* (5) was used for the color development.

Allen's method of correcting for impurities is applicable only when the absorption of the impurity is a linear function of the wave length. That this is a good approximation in this case may be seen from curve NK in

Figure 1. The slight shoulder at 520 mμ is probably due to incomplete removal of ketones.

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The Endocrine Society

THE SEVENTH POSTGRADUATE ASSEMBLY IN ENDOCRINOLOGY AND METABOLISM

SPONSORED JOINTLY BY THE ENDOCRINE SOCIETY AND THE INDIANA UNIVERSITY SCHOOL OF MEDICINE AT THE CONTINUATION STUDY CENTER OF THE INDIANA UNIVERSITY MEDICAL CENTER, INDIANAPOLIS, INDIANA

September 26–October 1, 1955

The faculty will consist of twenty-one eminent clinicians and investigators from various parts of the country in the fields of endocrinology and metabolism. The program will cover the various endocrinopathies, with emphasis on the clinical aspects, demonstration of laboratory tests, presentations of cases, and question-and-answer panel discussions. The course is designed to cover the main aspects of diagnosis and therapy in the field of endocrinology and metabolism for the physician in general practice and for those in other specialties who wish to have a general knowledge of this rapidly growing field.

A syllabus with brief abstracts of lectures will be available to the registrants at the time of the assembly.

For further information concerning the program, write Post Graduate Office, Indiana University Medical Center, 1100 West Michigan Street, Indianapolis 7, Indiana. Registration is limited to 100; tuition fee is $100.00. Arrangements for housing accommodations will be made through the Indiana Medical Center.

THE 1956 AWARDS

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

MEDAL OF THE ENDOCRINE SOCIETY

In 1954 the Council of the Society voted to establish a medal and an honorarium of $1,000 to be given to an individual for work of special distinction in endocrinology. The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada.

THE CIBA AWARD

The Ciba Award, to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945—Dr. Jane A. Russell; 1946—Dr. Martin M. Hoffman; 1947—Dr. Choh Hao Li; 1948—Dr. Carl Heller; 1949—Dr. George Sayers; 1950—Dr. Oscar M. Hechter; 1951—Dr. Albert Segaloff; 1952—Dr. Seymour Lieberman; 1953—Dr. Sidney Roberts and Dr. Clara Szego (Mrs. Roberts); 1954—Dr. Isadore M. Rosenberg. Prior to 1952 the Award was $1,200. It is now $1,800. If within twenty-four months of the date of the award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, it will be increased to $2,500.

THE AYERST, McKENNA AND HARRISON FELLOWSHIP

This Fellowship was established in 1947. It is designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. The Fellowship is awarded on alternate years (1955, 1957, etc.) and the stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

THE SCHERING AND THE UPJOHN SCHOLARS OF THE ENDOCRINE SOCIETY

The Council of The Endocrine Society has established a category of Scholars. These grants have been made available through the generosity of

the Schering Corporation and the Upjohn Company, and will be awarded to established investigators and teachers in the field of endocrinology who wish to extend their opportunities for work either in this country or abroad.

The awards will not exceed $2,500 annually for each individual and will be granted on the basis of proposals submitted by the applicant. Such applications should include the estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Medal of the Endocrine Society; the Ciba Award; and the Ayerst, McKenna and Harrison Fellowship may be made by any member of the Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than November 1 each year.

Proposals for appointments as Scholars of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by November 1 each year.

The Awards Committee will meet in November and notice of awards to successful nominees and applicants will be made not later than December 1.

ENZYME SYMPOSIUM

A 3-day International Symposium on ENZYMES: UNITS OF BIOLOGICAL STRUCTURE AND FUNCTION, sponsored by the Henry Ford Hospital and The Edsel B. Ford Institute for Medical Research will be held in the auditorium of Henry Ford Hospital, November 1, 2, and 3, 1955.

Interrelationships between enzymology and other fields, notably genetics, physiology, biochemistry and pharmacology, will constitute the general theme of the Symposium. The specific topics for the six sessions will be: Origin of Enzymes, Status of the Gene-Enzyme Relationship, Enzymes and Cell Structure, Enzymatic Basis of Some Physiological Functions, Cellular Energy Sources, and Regulation of Enzyme Activity. More than thirty internationally known scientists have accepted invitations to participate.

Interested persons may secure a copy of the Preliminary Announcement by writing to Dr. Clarence E. Rupe, Henry Ford Hospital, Detroit 2, Michigan.

Invitations will be sent to as many as can be accommodated.

BOOKS RECEIVED

Diseases of the Thyroid Gland. By SAMUEL L. GARGILL, M.D., Assistant Clinical Professor of Medicine, Harvard Medical School; Visiting Physician, Consultant to Endocrine Clinic, and Head of the Fertility Clinic, Beth Israel Hospital, Boston, Mass., and MARK FALCON LESSES, M.D., Instructor in Medicine, Harvard Medical School; Visiting Physician, Chief of Endocrine Clinic, and Director of Blood Bank, Beth Israel Hospital, Boston, with a Foreword by J. H. MEANS; newer aspects of the biochemistry and physiology of the thyroid are reviewed; the interrelations of the thyroid with other endocrine glands are discussed at length; the current place of antithyroidal agents in the treatment of thyrotoxicosis is elaborated; the metabolism of iodine and its relation to the structure and the function of the thyroid are described; all the usual and some less usual clinical syndromes are presented, and finally the insight into physiologic and clinical phenomena afforded by radioactive iodine is repeatedly utilized; (reprinted from Oxford Loose-Leaf Medicine); 509 pages; 68 figures; 9 plates; 1955. New York, Oxford University Press. Price $12.00.

Methods for Evaluation of Nutritional Adequacy and Status. A Symposium Sponsored by the Quartermaster Food and Container Institute for the Armed Forces Quartermaster Research and Development Command, U. S. Army Quartermaster Corps, and Medical Nutrition Laboratory, Research and Development Division, Office of the Surgeon General, Oriental Institute, University of Chicago, February 25–26, 1954. Edited by HARRY SPECTOR, MARTIN S. PETERSON and T. E. FRIEDEMANN. Preface by HERBERT POLLACK, Consultant on Nutrition to The Quartermaster General and The Surgeon General, U. S. Army; 34 papers and discussions by authorities in the field of nutrition; 320 pages; December 1954. Copies are available (without charge) at the Quartermaster Food and Container Institute for the Armed Forces, 1819 West Pershing Road, Chicago 9, Illinois.

Practical Endocrinology. By LEWIS M. HURXTHAL, M.D., Head of the Department of Internal Medicine, Lahey Clinic, Boston, Mass., in cooperation with A. SEYMOUR PARKER, M.D., Physician, Department of Internal Medicine, Lahey Clinic, and HIRSH SULKOWITCH, Research Associate, Lahey Clinic. A Handbook for the General Practitioner. After the various disorders of each gland are described along with method of diagnosis and treatment, several special chapters are presented, dealing with hirsutism, gynecomastia, sterility, and therapeutic uses of steroid compounds, corticotropin and chorionic gonadotropin. There is also a chapter on simple laboratory procedures; 330 pages; 17 figures; 1955. New York, The Blakiston Division of the McGraw-Hill Book Co. Price $7.00.

Should the Patient Know the Truth? A response of physicians, nurses, clergymen, and lawyers; edited by SAMUEL STANDARD, M.D. and HELMUTH NATHAN, M.D.; 160 pages; 1955. New York, Springer Publishing Co., Inc. Price $3.00 (hard cover); $2.00 (soft cover).

Studies on Fertility. Edited by R. G. HARRISON, M.A., D.M., Derby Professor of Anatomy in the University of Liverpool, England. Volume VI of the Proceedings of The Society for the Study of Fertility, including papers read at the Conference of the Society, London, 1954; 160 pages; 64 illustrations; 19 tables; 1955. Springfield, Illinois, Charles C Thomas, Publisher. Price $4.25.

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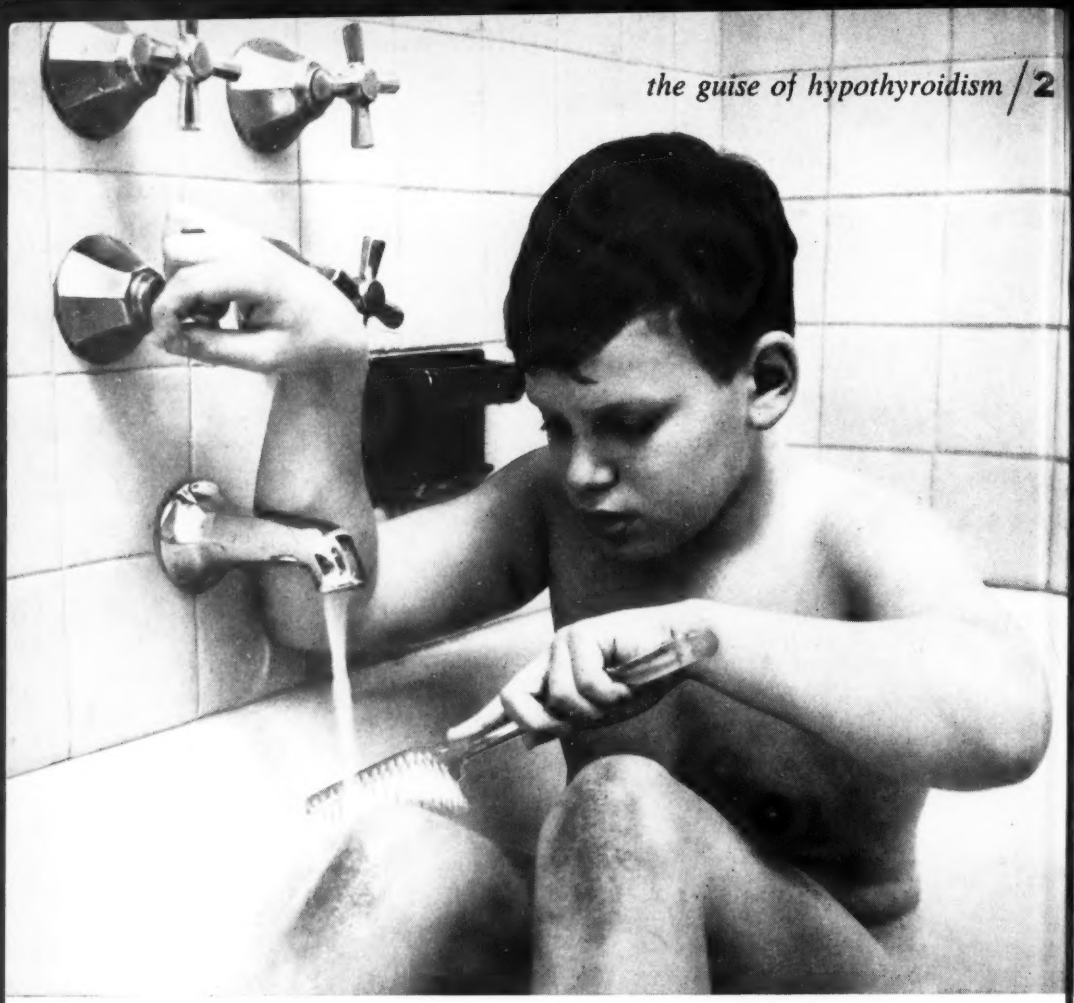

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